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# Activity patterns of central amygdala neurons in a mouse model of narcolepsy

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**ACTIVITY PATTERNS OF CENTRAL AMYGDALA NEURONS IN A MOUSE  
MODEL OF NARCOLEPSY**

by

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B.A., Wellesley College, 2017

Submitted in partial fulfillment of the  
requirements for the degree of  
Master of Science

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# **ACTIVITY PATTERNS OF CENTRAL AMYGDALA NEURONS IN A MOUSE**

## **MODEL OF NARCOLEPSY**

**JELENA BEGOVIĆ**

### **ABSTRACT**

Narcolepsy is a disorder of unstable wake and sleep states caused by the lack of orexin neurons which degenerate most likely as a consequence of an autoimmune process. The state instability of narcolepsy includes rapid eye movement (REM) sleep intruding into wake in the form of dream-like hallucinations and cataplexy, muscle paralysis (atonia) much like occurs in REM sleep. In mice lacking orexin peptides, cataplexy is also observed with similar presentation as in humans of muscle paralysis during wakefulness which is often triggered by positive emotions. Prior research showed that the activation of the central amygdala is sufficient to promote cataplexy in a mouse model of narcolepsy. The central amygdala (CeA) contains a variety of neuronal types, and we hypothesize that  $\gamma$ -aminobutyric acid (GABA)-ergic neurons expressing the oxytocin receptor (OTR) mediate cataplexy as these neurons project to a known REM sleep atonia-regulating region, the ventrolateral periaqueductal gray (vlPAG)/lateral pontine tegmentum (LPT), and, as oxytocin (OT) sensitive neurons in the amygdala, likely participate in emotional processing and social behavior. In this study, we used fiber photometry to investigate the behavior of these neurons in response to social and rewarding stimuli, during emotion-triggered cataplexy, and across arousal states in an effort to define their potential role in emotion-triggered cataplexy. Initial recordings were conducted at too low an excitation light power to stimulate the green fluorescent calcium

indicator, GCaMP6s, but were useful in optimizing MATLAB analysis and behavioral tests later done at higher LED power. The second series of recordings with higher excitation light power and better signal to noise ratio, showed increased activity in response to social interaction and reward, prior to REM transitions, and decreased activity during cataplexy confirming patterns seen in initial recordings. In recordings with higher excitation light, these responses appear to occur before interaction with stimulus mice or reward stimulus. In the future, additional recordings with a higher signal to noise ratio will be needed to confirm these results. In conclusion, responses of CeA-OTR neurons to social and rewarding stimuli, cataplexy, and at REM transitions are in support of a possible role of these neurons in emotion-triggered cataplexy which can be tested using additional methods, such as optogenetics.

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## LIST OF ABBREVIATIONS

AAV	Adeno-Associated Viral vector
AP	Anterior/Posterior
aq	Aqueduct
CeA	Central Amygdala
cre	Gene name: cyclization recombinase
CRE	Protein name: CRE Recombinase (Sauer 1994; Tsien 2016)
DAB	3,3'-diaminobenzidine
DIO	Double-Floxed Inverted Open Reading Frame
DNA	Deoxyribonucleic acid
DV	Dorsal/Ventral
EEG	Electroencephalogram
EMG	Electromyogram
GABA	$\gamma$ -aminobutyric acid
GCaMP6s	Green Fluorescent Protein with Calmodulin and M13, a Myosin Light Chain Kinase, Slower Kinetics
GFP	Green Fluorescent Protein
HLA	Human Leukocyte Antigen
LC	Locus Coeruleus
LH	Lateral Hypothalamus
LPT	Lateral Pontine Tegmentum

ML	Medial/Lateral
NHS	Normal Horse Serum
nREM	Non-Rapid Eye Movement
NT1	Narcolepsy Type 1
NT2	Narcolepsy Type 2
OT	Oxytocin
OTR	Oxytocin Receptor
OXKO	Orexin Knock-Out
PBS	Phosphate Buffered Saline
PBT	Phosphate Buffered Solution and Tween
PPT	Pedunculopontine Tegmental Nucleus
REM	Rapid Eye Movement
SEM	Standard Error of the Means
SLD	Sublaterodorsal Nucleus
vIPAG	Ventrolateral Periaqueductal Gray

## INTRODUCTION

### *Narcolepsy*

Narcolepsy is a sleep disorder characterized by excessive sleepiness. While sleepiness is the major symptom, patients do not actually sleep more – instead, they have unstable wake-sleep states, resulting in more frequent transitions between states and a poor ability to maintain long periods of wakefulness and sleep (Mochizuki et al. 2004; Sorensen, Knudsen, and Jennum 2013; Ferri et al. 2005).

There are two types of narcolepsy. Type 1 (NT1) is associated with very low cerebrospinal fluid levels of the orexin neuropeptides (also known as hypocretins) and tends to have more severe symptoms, whereas Type 2 (NT2) can have normal orexin levels, milder symptoms, and tends to be more difficult to diagnose (Hansen, Kornum, and Jennum 2017). NT1 is caused by loss of the orexin-producing neurons (Peyron et al. 2000; Thannickal et al. 2000; Crocker et al. 2005). The orexin neuropeptides are wake-promoting, and their loss in narcolepsy causes an inability to maintain wakefulness and contributes to lower threshold to transition between wake-sleep states (Eggermann et al. 2003; Estabrooke et al. 2001).

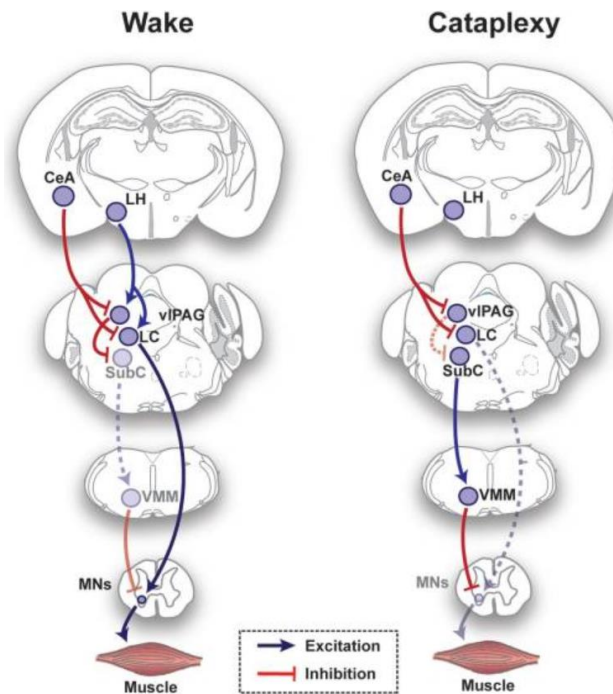
The cause of the loss of orexin neurons is unclear but likely a combination of genetic, autoimmune, and age-dependent factors. Age is implicated as patients tend to experience onset of symptoms in youth between 10 and 30 years of age. A genetic risk factor for development of narcolepsy has been identified; human leukocyte antigen (HLA) gene DQB1\*0602 is present in over 90% of narcolepsy patients (Mignot et al. 1994). Increased incidence of narcolepsy following H1N1 influenza vaccination supports

the hypothesis that narcolepsy is caused by an autoimmune response, possibly incited by an inflammatory response in individuals with HLA DQB1\*0602 (Dauvilliers et al. 2010). Recently, CD4+ T cells were discovered that recognize orexin neuropeptides in narcolepsy patients, and thus may allow these CD4+ T cells to target orexin neurons, supporting this autoimmune hypothesis (Latorre et al. 2018).

In addition to sleep-wake instability, symptoms of narcolepsy include disrupted rapid eye movement (REM) sleep patterns. REM sleep is characterized by high frequency, low-amplitude electroencephalogram (EEG), muscle atonia, and dreaming. It usually occurs following non-rapid eye movement (nREM) sleep. In people, REM sleep occurs only during the usual nighttime sleep period, but in narcolepsy, REM sleep can occur at almost any time of day including during short naps. In addition, elements of REM sleep can intrude into wake and are believed to be responsible for symptoms like sleep paralysis, and hypnagogic hallucinations. Sleep paralysis is REM sleep muscle atonia occurring during early waking and can be quite frightening. Hypnagogic hallucinations are auditory or visual hallucinations that typically occur when someone is very sleepy and/or falling asleep. Cataplexy is described as another REM-like symptom, only present in NT1, that exhibits similar EEG pattern as well as full-body muscle atonia as occurs during REM sleep. The difference between cataplexy and REM sleep is that a person is fully awake and aware during an episode of cataplexy. Cataplexy episodes can last multiple seconds to minutes, can have partial or full muscle atonia, and are often triggered by positive emotions.

### ***Cataplexy Circuit***

These parallels to REM sleep suggest that cataplexy arises from the same neural circuitry. Neurons in the central amygdala (CeA) and orexin neurons in the lateral hypothalamus (LH) both participate in regulation of REM atonia through their projections to the ventrolateral periaqueductal gray (vlPAG), locus coeruleus (LC) and pedunculopontine tegmental nucleus (PPT) (Boissard et al. 2003). Most CeA neurons are inhibitory and orexin neurons are excitatory. Researchers hypothesize that during normal wakefulness, excitation of the vlPAG/lateral pontine tegmentum (LPT) by the orexin neurons is greater than inhibition by CeA neurons. Thus, the vlPAG/LPT is excited and inhibits the sublaterodorsal nucleus (SLD) preventing atonia (Lu et al. 2006). Additional brain regions may contribute to maintenance of muscle tone, including the LC. In the absence of excitation by orexin neurons, the LC is not excited and the SLD is not inhibited enabling the glutaminergic neurons in the SLD to excite neurons in the ventromedial medulla which inhibit motor neurons resulting in muscle atonia during wake (Wu et al. 1999). Thereby, completing a circuit in which the CeA would initiate muscle atonia as occurs with cataplexy (Figure 1) (Lai and Siegel 1988; Burgess and Scammell 2012). The amygdala is involved in emotion processing as described for negative valence and positive valence stimuli (Hugh et al. 2001; Davis 1992; Paton et al. 2006). Lesion of the amygdala or inhibition of the CeA reduces cataplexy in the presence of rewarding stimuli (Burgess et al. 2013; Mahoney et al. 2017). Rewarding and emotional stimuli promote cataplexy in humans (Overeem et al. 2011; Phelps and LeDoux 2005). Thus, some researchers postulate that neurons in the CeA likely promote cataplexy.



**Figure 1 REM Muscle Atonia Circuit during Wake and Cataplexy** In absence of excitation by orexin neurons in narcolepsy CeA neurons inhibit vIPAG and LC, allowing the SubC (SLD) neurons to promote muscle atonia (Fragne et al. 2015)

### ***Social and Rewarding Behavior***

In mice as well, cataplexy is more frequent in presence of reward or positive emotion caused by palatable food, running wheel activity, or social interaction (España et al. 2007; Meletti et al. 2015; Clark et al. 2009; Phelps and LeDoux 2005). While social interaction may not always be rewarding, for example in cases of aggression, social interaction can be rewarding to mice such as during play behavior (Calcagnetti and Schechter 1992). In fact, punishment and reward are present in almost all cases of social approach and withdrawal (Glickman and B. Schiff 1967).

Social interaction is mediated in part by the neurotransmitter, oxytocin (OT) (Ross and Young 2009). Oxytocin is required for social reward, and promotes sociability

(Hung et al. 2017; Dong et al. 2017). OT in the limbic system, including the central amygdala, is involved in maternal behavior and positive reinforcement (Stamatakis et al. 2015; Laszlo et al. 2016). Social behavior can vary with genetics; for example, mutations of the oxytocin receptor (OTR) gene can affect human behavior resulting in autism (Panksepp et al. 2007; Moy et al. 2004; Saito et al. 2014; Takayanagi et al. 2005). OT mediates amygdala response to positive and negative emotion in response to social interaction (Domes et al. 2007). The amygdala participates in processing reward and punishment, and stimulation of the CeA increases reward-seeking behavior (Namburi et al. 2015; Warlow, Robinson, and Berridge 2017). Some CeA neurons express OTR and these neurons are thought to promote social interaction (Dumais et al. 2016).

### ***CeA-OTR Neurons***

CeA-OTR neurons are a subpopulation of  $\gamma$ -aminobutyric acid(GABA)-ergic neurons in the CeA (Huber 2005; Nakajima, Görlich, and Heintz 2014). The GABAergic neurons of the CeA project to known REM sleep-regulating regions, including the periaqueductal grey (PAG) and PPT (Burgess and Scammell 2012). Different populations of neurons in the CeA can increase or decrease muscle tone in different contexts. Projections of the CeA on the PAG, hypothalamus and brain stem nuclei promote freezing in response to fear (LeDoux et al. 1988; Viviani et al. 2011; Haubensak et al. 2010). Somatostatin-expressing neurons in the lateral CeA are sufficient to cause a freezing response to fear (Yu et al. 2016). OT activates a subpopulation of GABAergic neurons in the CeA that inhibit the muscle tone promoting neurons of the CeA thereby reducing the freezing response to fear (Knobloch et al. 2012). In addition, GABAergic

neurons in the CeA promote cataplexy (Mahoney et al. 2017). Because CeA-OTR neurons are OT-sensitive, they may also be involved in social interaction and therefore provide a link between social interaction and positive emotion and cataplexy.

Understanding whether they are active before or at the start of social interaction, reward approach, sleep-wake state transitions as well as how active these cells are during different arousal states will determine whether these cells are more likely to be involved in decision-making, initiation, or maintenance. We will be looking for patterns of activity of CeA-OTR neurons in mice during social and rewarding stimuli, cataplexy, and sleep-wake state transitions to elucidate their potential role in emotion-triggered cataplexy.

## **OBJECTIVES**

1. To use fiber photometry recordings of CeA-OTR neurons in mice to determine patterns of activity in response to social interaction in the Home Cage Social Test and U-Chamber Social Test.
2. To determine patterns of activity of CeA-OTR neurons in response to rewarding stimuli.
3. To determine patterns of activity of CeA-OTR neurons around cataplexy onset and offset during social stimuli, different reward conditions, and spontaneous cataplexy.
4. To determine patterns of activity of CeA-OTR neurons around sleep-wake state transitions and mean levels of activity across states.
5. To compare patterns of activity of CeA-OTR neurons around cataplexy transitions to form hypothesis for how these cells participate in emotion-triggered cataplexy.

## METHODS

### *Animals*

The following protocols were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center and Harvard Medical School. All experiments were performed in accordance with the *National Institutions of Health Guide for the Care and Use of Laboratory Animals*

In this experiment, we used orexin knock-out (OXKO) mice which are homozygous for the null-pre-pro orexin gene meaning they lack orexin peptides which make them a good model of NT1 exhibiting both instability of sleep states and cataplexy (Chemelli et al. 1999). To perform photometry recordings of CeA-OTR neurons in response to cataplexy, we used a mouse model of narcolepsy that enables gene expression selectively in neurons expressing OTR. OXKO mice were crossed with OTR-cre mice in which the OTR promoter drives expression of the cyclization recombinase (cre) gene. CRE recombinase, the protein coded for by cre, is a deoxyribonucleic acid (DNA) recombinase that recombines a pair of target sequences, lox sequences, allowing for manipulation of gene expression. When adeno-associated viral vector (AAV)-double-floxed inverted open reading frame (DIO)-green fluorescent protein+calmodulin+M13 with slow kinetics (GCaMP6s) is injected into the CeA, the GCaMP6s will be expressed only in those cells that (a) are transduced with the viral vector and (b) express CRE recombinase, in this case OTR neurons. GCaMP6s is a calcium indicator that changes conformation and fluoresces in response to increases in calcium concentration. When a neuron increases activity, there will be a rise in intracellular calcium and an increase in

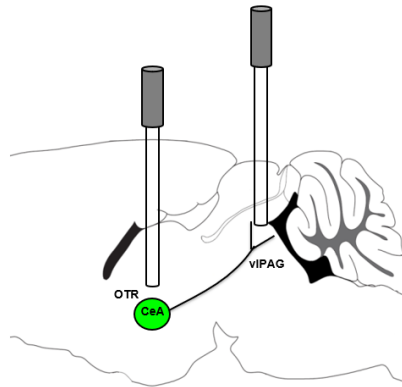
green fluorescence released from the GCaMP6s. Fluorescence of GCaMP6s during light excitation provided by a low power LED (0.1nW) (later using 0.2 $\mu$ W), was measured using a single photodetector for each optical fiber. Sampling rate was 256Hz. A beam splitter/combiner was used to separate excitation wavelength (blue 470nm) from green fluorescent protein (GFP) fluorescence (green 395nm).

All animals were singly housed on a 12/12 light/dark cycle (lights on at 0530) with constant temperature ( $22 \pm 1.6^{\circ}\text{C}$ ) and humidity ( $25 \pm 2.2\text{mmHG}$ ). Regular chow and water were available *ad libitum*.

Stimulus mice for social tests were age- and sex-matched. Chow was restricted overnight prior to reward tests. No tests were conducted for 72 hours after cages were cleaned to avoid any effects related to stress.

### ***Surgery***

The OXKO-OTRcre mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.) and placed in a stereotaxic alignment system (model 1900; Kopf Instruments). All mice were unilaterally injected with 90nL of AAV-DIO-GCaMP6s using an air pressure injection system and glass micropipette (~20 $\mu$ m tip diameter) targeted at the CeA (anterior/posterior (AP): +1.5mm, medial/lateral (ML): -2.8mm, dorsal/ventral (DV): -4.7mm from bregma). All mice were fitted with EEG/electromyogram (EMG) head stages and optical fibers were implanted just above the CeA (AP: +1.5mm, ML: -2.8mm, DV: -4.5mm from bregma) and the vIPAG (AP: +4.2mm, ML: -0.8mm, DV: -2.8mm from bregma). Each mouse was treated with meloxicam slow release form (4mg/kg, subcutaneously) immediately after surgery.



**Figure 2 Diagram of Optical Fiber Placement.** Fibers were placed to record both CeA-OTR neuron cell bodies in the CeA and associated terminals in the vIPAG.

### ***Experimental Design: Behavioral Tests***

Testing began 4 weeks post-surgery to enable expression of high levels of GCaMP6s. Following habituation to the cable plugged into the head stage for 72 hours, mice underwent three different tests, a Home Cage Social Test (n = 2), U-Chamber Social Test (n = 6), and Reward Test (n = 6). Traces were recorded with corresponding EEG/EMG and video, and scored for wake, sleep, or cataplexy state. Baseline overnight recording was also scored (n = 6) in the mouse's home cage with food and water *ad libitum*.

Photometry cables are more fragile than the EEG/EMG cable and were connected just prior to testing to prevent damage to the fibers. The first five minutes of photometry recordings were excluded to account for bleaching by excitation light. Bleaching follows an exponential decay pattern, and by excluding the first five minutes we ensured a more stable baseline. Photometry analysis was performed in MATLAB.

### *Home Cage Social Test*

This social test was performed in the mouse's home cage and included 20 minutes with a stimulus mouse, and 20 minutes with a toy sham mouse with 10-minute sessions without either stimulus mouse or sham mouse in the cage to separate the trials. Order of introduction of stimulus or sham mouse was randomized.

Recordings were scored for 3 types of social interaction depending on which mouse appeared to initiate the interaction, subject or stimulus mouse, or mutual when an initiator was unclear. Start of social interaction was defined as direct contact with the stimulus mouse. An approach towards the sham mouse was defined as direct contact with the toy.

### *U-Chamber Social Test*

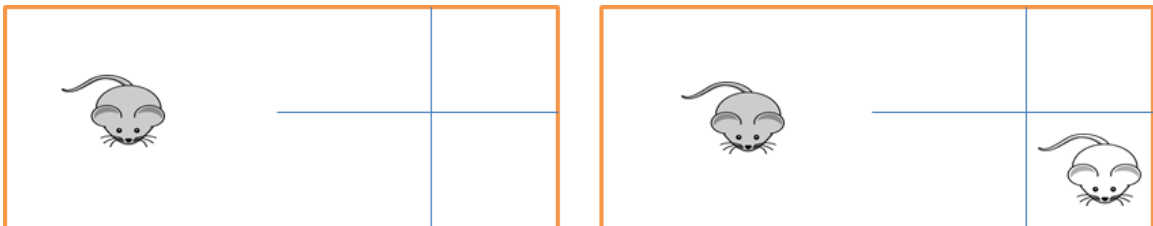
The U-Chamber Social Test was conducted within a rectangular chamber that has three chambers, two adjacent chambers separated by a wall that each contain smaller chambers separated by another wall with a square mesh window about 3in x 3in opening, and a neutral chamber that allows access to the other two chambers (Figure 3). The walls of the chamber are all white. The test chamber was placed within the cabinet where mice are normally housed with the mesh window chambers near the cabinet doors for easy access.

Once the test mice were attached to the photometry cables, they were placed in the neutral chamber and recorded moving freely for 15 minutes. Then, a stimulus mouse was placed behind either one of the mesh windows as recording proceeds for another 15 minutes.

Two age- and sex-matched stimulus mice were altered across tests as was the mesh window behind which they were placed. The chamber in which the stimulus mouse was placed was designated the social chamber and remaining chamber was designated as the empty chamber.

Unlike in the Home Cage Social Test, social interaction in the U-Chamber can only be initiated by the subject mouse as the stimulus mouse is confined, and any negative behavior such as following, where a mouse sniffs and follows another, would be impossible. Events recorded include when the subject enters either chamber, or approaches either mesh both pre- and post-stimulus mouse addition.

Between tests, the chamber was cleaned of urine and feces and washed with 70% ethanol and clidox for 7 minutes. At a minimum, a mouse spent 45 minutes in its home cage before consecutive U-Chamber Social Tests.



**Figure 3 U-Chamber for Social Test** The U-Chamber with an open (neutral) chamber, and two smaller chambers separated by a wall. A stimulus mouse (white) is placed in one of the two smaller chambers behind a mesh after 15 minutes of free movement about the chamber.

### *Reward Test*

This test, like the Home Cage Social Test, was conducted in the mouse's home cage. The mice were food-deprived overnight before testing. After connection to photometry cables, recording began, and mice were given 5 minutes before addition of the first reward item. Rewards were a small piece of a Hershey's Kiss chocolate (a highly

palatable food), followed by a marble (a novel object), followed by a small piece of regular chow. Each reward was present in the cage for 15 minutes, and the subject mouse was given 5 minutes between reward items. When rewards were placed in the cage, a small area was cleared, a circular area about 2 inches across, in the bedding so the reward item can be clearly seen on camera. An approach towards the reward item was defined as the mouse directly pointed at the item and within this cleared area.

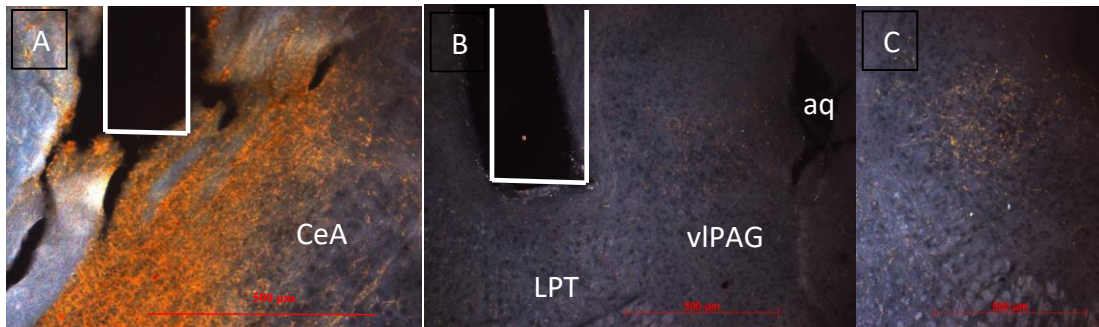
### ***Wake, Sleep, and Cataplexy Scoring***

The SleepSign (Kissei Comtec) program was used for scoring the EEG/EMG and video recording of each test trial and the overnight recordings (filter settings: EEG, 0.25-64Hz; EMG, 10-60Hz). Recordings were divided into 10s epochs and each epoch was labelled as either wake, nREM, REM, or cataplexy corresponding to what state takes up most of that epoch. Low amplitude, fast EEG and high EMG, often with mouse movement confirmed in video was scored as wake; slow-wave high amplitude EEG with low EMG and curled up/tucked tail position confirmed in video was scored as nREM sleep; and more theta frequency, 4-9Hz, low amplitude EEG with very low EMG, and sleeping position confirmed in video was defined as REM sleep. Cataplexy was defined with similar EEG/EMG to REM sleep, onset must be preceded by at least 4 wake epochs and offset followed by a wake epoch. Cataplexy was confirmed using visual cues in video, including head drop at onset, legs splayed-tail untucked, falling over or forward, rocking, and/or popping up suddenly at cataplexy offset.

### ***Histology and Immunohistochemistry***

Once testing was complete, mice were deeply anesthetized with ketamine/xylazine (150/15 mg/kg) and transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 10% formalin. Brains were extracted and kept in 10% formalin. Before sectioning, brains were placed in 30% sucrose in PBS azide for at least 24 hours. Brains were sectioned on a freezing microtome at 30 $\mu$ m and collected in a 1:4 series in PBS-azide. Series were kept in cryoprotectant and stored at 4°C.

A series was immunostained for GFP to visualize the injection site. Sections were incubated overnight in chicken anti-GFP (Invitrogen, Product #A10262, Lot# 1812487), diluted 1:10000 in phosphate buffered solution and tween (PBT)-normal horse serum (NHS) solution. The next morning, after washing the sections in PBS 6 times for 5 minutes each, sections were incubated in biotinylated donkey anti-chicken antibody (Jackson ImmunoResearch Laboratories Inc., Product #203-065455, Lot# 117066) diluted 1:500 in PBT-NHS. Sections were incubated an hour in avidin-biotin complex before staining brown with 3,3'-diaminobenzidine (DAB). Sections were mounted on glass slides.



**Figure 4 Injection Site and Optical Fiber Placement** Example of confirmed injection and optical fiber placement in CeA (A), and vIPAG/LPT (B). White lines outline optical fiber tract. Image (C) shows how fibers can look. They are difficult to see in (B) because the plane of section did not capture the longitudinal axis of the fibers, rather the coronal plane. Central Amygdala (CeA), Lateral Pontine Tegmentum (LPT), ventrolateral periaqueductal gray (vIPAG), Aqueduct (aq)

### ***Confirming Injection Sites and Optical Fiber Placement***

Injection sites and optical fiber placement were mapped using dark-field images of DAB stained sections. Injections were considered successful when >75% of cell bodies were stained in the CeA, and optical fiber tracks could be seen to extend within 1mm of the center of the CeA and within 1mm near the vIPAG to the LPT (Figure 4). Fiber tracts that were near the LPT in addition to vIPAG were accepted as both regions have been shown to regulate REM atonia (Lu et al. 2006). Where vIPAG is referred to, some of those signals are also from terminals in the LPT. If the fiber tract was distant from the target of interest, or staining was present outside the CeA, these were considered misses and not included in analysis.

### ***Statistical Analysis***

Photometry traces were smoothed to reduce noise in MATLAB, and the raw traces were converted to dF/F traces where  $dF/F = (f_i - f_0) / f_0$ . Baseline fluorescence ( $f_0$ ) was defined as the 10<sup>th</sup> lowest percentile value in the 20s preceding the event. All plots of

photometry traces are shown with standard error. All means of activity are shown with standard error of the means (SEM). Means were compared using 2-sample *t*-tests with two-tailed  $\alpha$  value of  $p < 0.05$ . Pearson's correlation coefficient was also used to compare traces.

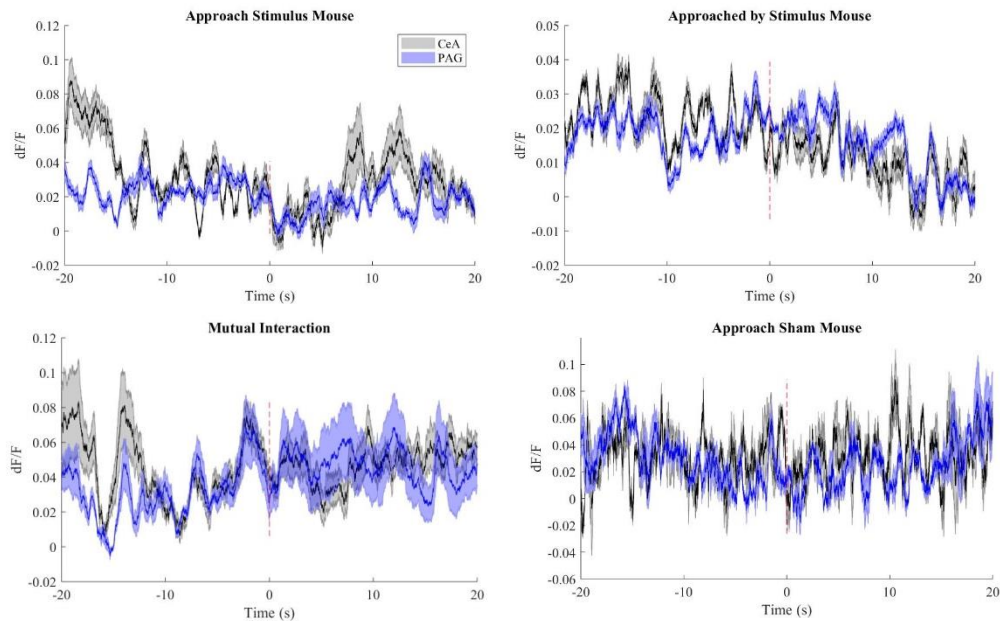
Traces where injection site of optical fiber placement was not confirmed were excluded, as were traces where there was too much noise or artifact to be physiologically possible defined as vertical changes in  $dF/F$  ( $> 75.75 dF/F \cdot s^{-1}$ ) or sudden extremely high activity amplitude ( $>3 dF/F$ ), for example, caused by loose cables (Chen et al. 2013). We excluded 11.5% of our data based on these criteria. Optimal recording time for GCaMP6s is about 4-6 weeks post-surgery. Although recordings can be consistent after this period, a rising baseline activity level can be of concern. We had no traces with an increased baseline greater than 10%, with the majority under 1% change. Recordings were acquired 5-10 weeks post-surgery.

## RESULTS

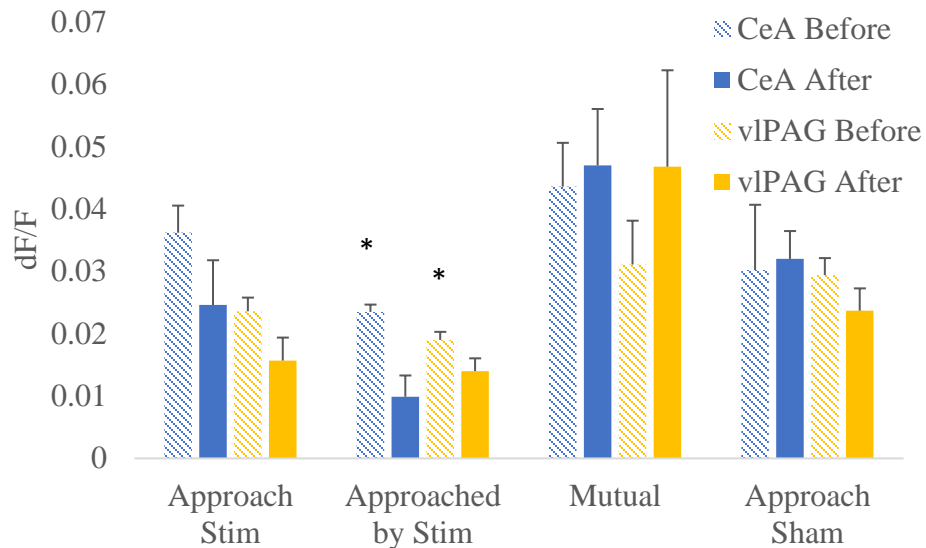
### *Home Cage Social Test*

During the Home Cage Social Test, activity of CeA-OTR neurons in the CeA and their terminals in the vIPAG/LPT were measured around interactions between the subject and stimulus mouse, both freely moving around the cage. These pilot results were taken from only 2 mice, 3 trials each at very low excitation light output power (0.1nW). When the subject mouse approached the stimulus mouse, there was increased activity ( $\sim 0.04 dF/F$ , Figure 5) just prior to approach, with peaks ( $\sim 0.06 dF/F$ ) about 8-15s following

initial interaction. An approach of the subject mouse by the stimulus mouse showed a decreasing trend in activity after interaction (Figure 5). In fact, mean activity following an approach by the stimulus mouse was significantly lower than before the approach in both the CeA and vIPAG (Figure 6). During a mutual interaction, there was increased activity ( $\sim 0.08$  dF/F) about 2s before the interaction (Figure 5). Activity following a mutual interaction was consistently around 0.05 dF/F; prior to interaction, there was high amplitude activity  $>10$ s before event, followed by activity around 0.03 dF/F (Figure 5). A mutual interaction, also, exhibited higher standard error of vIPAG signal following event, and greater standard error compared to vIPAG signals during other social events in the Home Cage Social Test (Figure 5). An approach of the sham mouse showed increased activity ( $\sim 0.06$  dF/F) just before the event in the CeA and vIPAG, with increased activity at least 10s before and after the event (Figure 5).

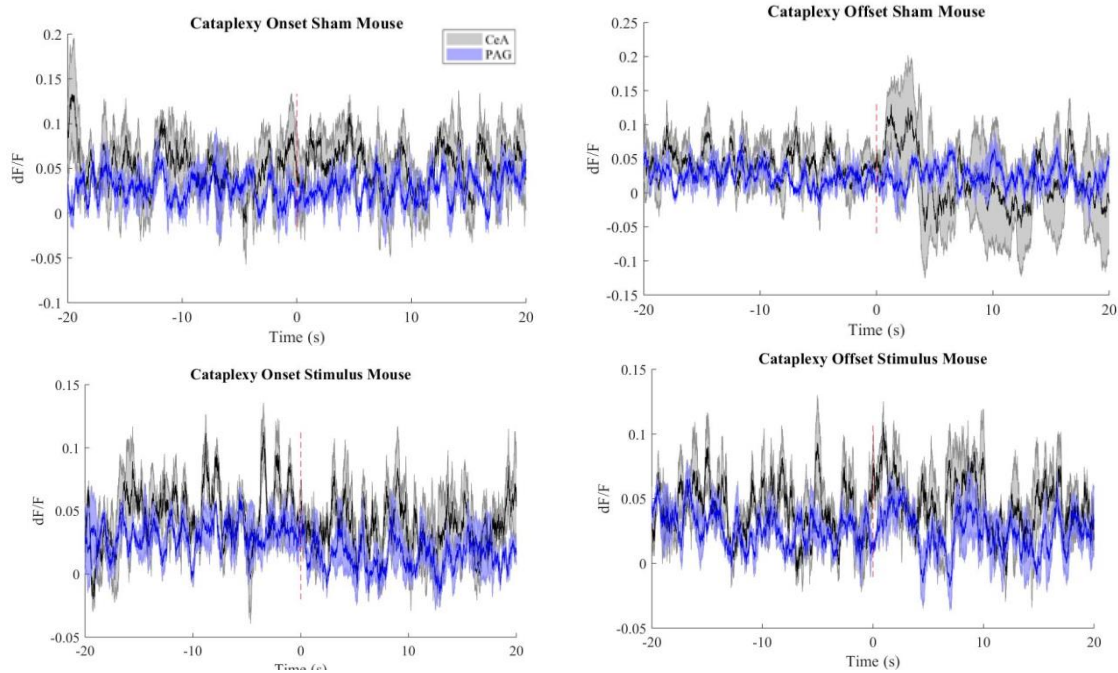


**Figure 5 Fiber Photometry Recordings of Home Cage Social Test.** Mean and standard error of photometry recording of CeA-OTR neurons in the CeA (gray) and vPAG (blue) shown for social events in Home Cage Social Test. Recorded from 2 mice, 3 trials each. Approach Stimulus Mouse ( $n = 39$ ) shows increase in CeA activity post event with a decrease right at zero. The vPAG signal is generally weaker than the CeA signal. Approached by Stimulus Mouse ( $n = 92$ ) shows decrease in  $dF/F$  post event, with smaller decrease in vPAG signal. Mutual Interaction ( $n = 33$ ) shows increase in activity near events and high variability in vPAG signal. Approach Sham Mouse ( $n = 5$ ) shows some increased activity prior to the event, but no clear trends.

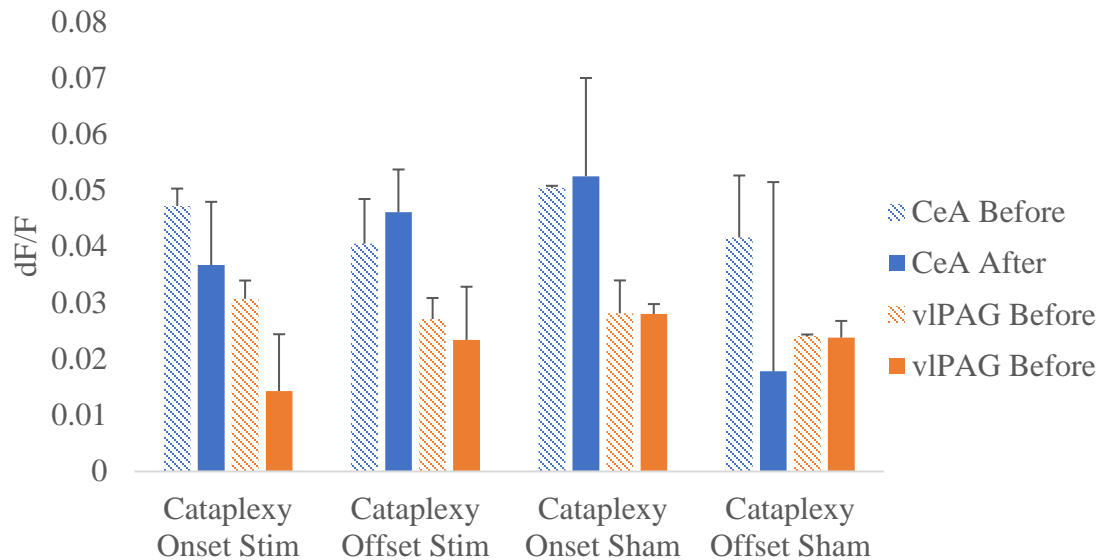


**Figure 6 Activity Before and After Home Cage Social Test Events.** Mean and SEM shown for 20s before and after events in the CeA and vIPAG. Approach Stim (n = 39), Approached by Stim (n = 92), Mutual (n = 33), Approach Sham (n = 5). Mean dF/F is significantly higher ( $p < 0.05$ ) before compared to after an approach by the stimulus mouse in both the CeA and vIPAG.

Cataplexy, during the Home Cage Social Test, occurred 6 times (4 in presence of stimulus mouse, 2 in presence of sham mouse). These results should be considered preliminary as the number of events is low. In presence of sham mouse, there was increased activity ( $\sim 0.08$  dF/F)  $-3$ - $5$ s around cataplexy onset, and abruptly increased activity ( $0.12$  dF/F) at offset, followed by a decrease in the CeA (Figure 7). The CeA signal was more variable following cataplexy offset with sham mouse (Figure 7, Figure 8). Similar patterns are not observed in the vIPAG signal (Figure 7). During cataplexy onset in presence of stimulus mouse, maximum activity ( $\sim 0.1$  dF/F) was observed up to  $5$ s before cataplexy onset, with a decrease in mean vIPAG activity following onset (Figure 7, Figure 8). Cataplexy offset with stimulus mouse showed increased activity in the CeA ( $\sim 0.08$  dF/F) and vIPAG ( $\sim 0.05$  dF/F) just after offset (Figure 7). There were no significant changes in mean activity before and after cataplexy transitions (Figure 8).



**Figure 7 Fiber Photometry Recordings of Cataplexy During Home Cage Social Test** Mean and standard error of photometry recordings of CeA-OTR neurons in the CeA (gray) and vPAG (blue) during Home Cage Social Test. Recordings are from 2 mice, 3 trials each. Cataplexy Onset and Offset in presence of Sham Mouse ( $n = 2$ ), and in presence of Stimulus Mouse ( $n = 4$ ) show similarities between CeA and vPAG recording with vPAG generally weaker. Increased dF/F near cataplexy onset both in presence of sham mouse and stimulus mouse. There is some increased activity post cataplexy offset, with high variability post cataplexy offset in presence of sham mouse.



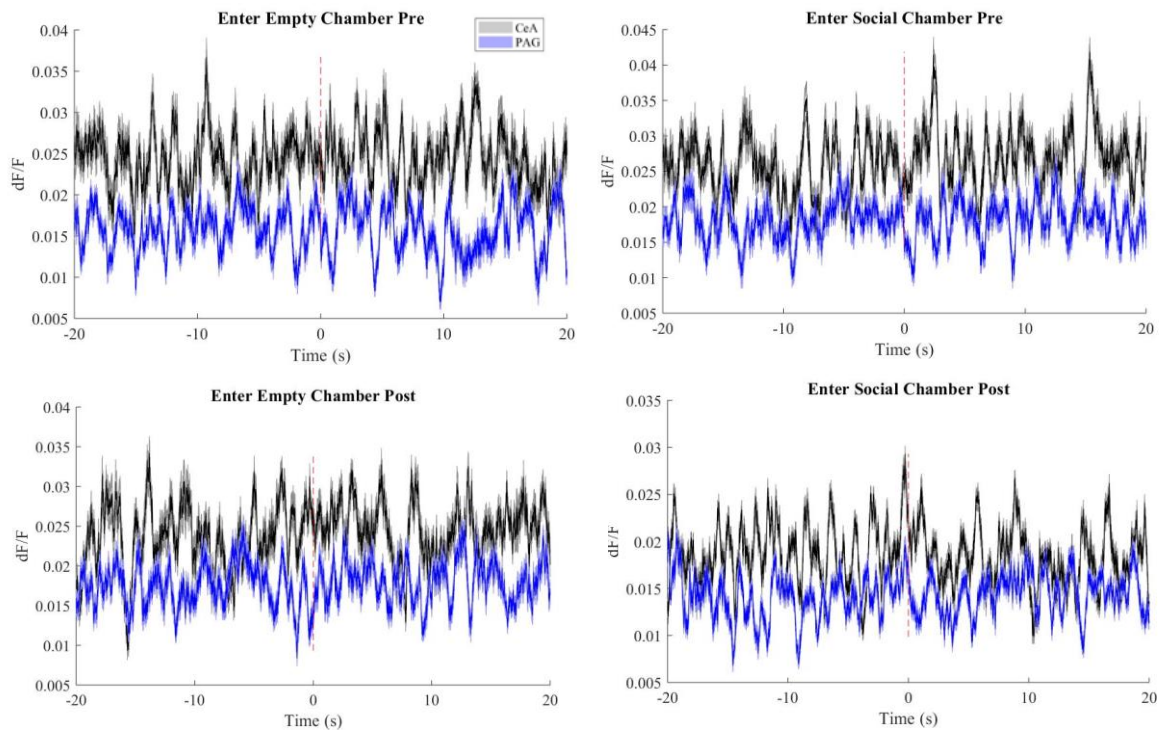
**Figure 8 Activity of Cataplexy Onset and Offset During Home Cage Social Test** Mean and SEM of dF/F from photometry recordings of CeA-OTR neurons in the CeA and vIPAG for 2 epochs (20s) before and after onset and offset of cataplexy in presence of sham mouse (n = 2) and in presence of stimulus mouse (n = 4). No significant sustained difference in activity at cataplexy onset and offset during Home Cage Social Test.

### ***U-Chamber Social Test***

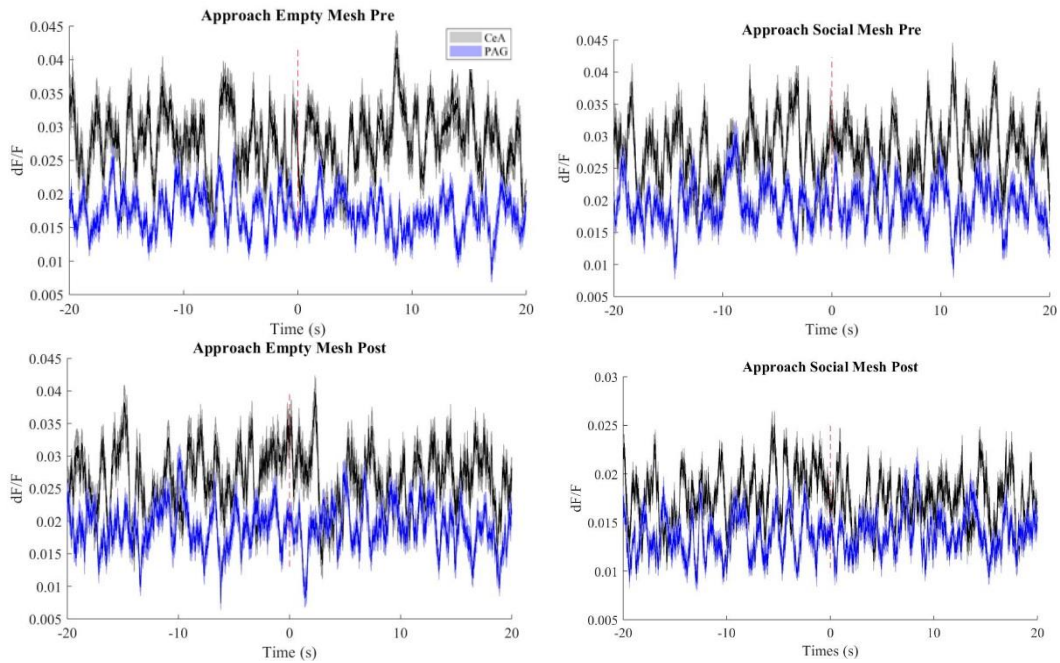
The U-Chamber Social Test is a social test conducted in a U-Chamber which restricts movement of the stimulus mouse and provides opportunities for the subject mouse to approach a social and non-social (empty) chamber. Activity of CeA-OTR neurons in the CeA and vIPAG/LPT was measured at low excitation light output power (0.1nW), complicated with some variation in power output at the vIPAG/LPT LED, as the subject mouse moved freely around the chamber. Before a stimulus mouse was added to the test chamber (Pre), when the subject mouse enters the empty chamber, activity was higher 5s following event with maximums an epoch before and after (Figure 9). When the mouse entered the social chamber, however, we saw maximum activity in the CeA 3s following event, with a similar peak 15s after event (Figure 9). When the mouse

approached the empty mesh and social mesh before the stimulus mouse was added (Pre), we saw similar increases in activity 2-8s before event, and about 10s following event in the CeA (Figure 10). After the stimulus mouse was added to the chamber (Post), when the mouse entered the social chamber, maximum activity in the CeA occurred at event (Figure 9). No such peak was present when the mouse entered the empty chamber (Figure 9). When the mouse approached the social mesh post and the empty mesh post there was increased activity around the event with maximum CeA activity at social mesh occurring about -5s, and at empty mesh at about +3s (Figure 10).

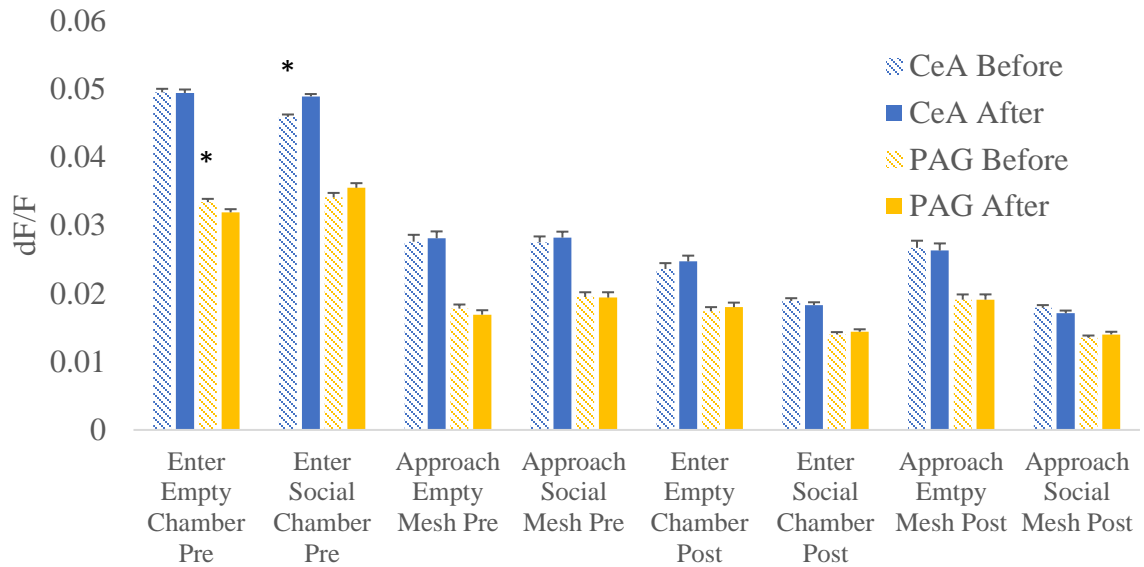
Mean activity around events in the U-Chamber Test showed no statistically significant differences except the vIPAG/LPT signal was significantly lower after entering the empty chamber pre, and the CeA signal was significantly higher after entering the social chamber pre (Figure 11). However, these are small changes, and looking at photometry recordings, we did not see sustained changes in activity around events (Figure 9, Figure 10). Analysis of maximum activity at event and just after (-1-5s) pre and post showed significantly decreased maximum activity post in the social chamber, but no significant changes in the empty chamber (Figure 12).



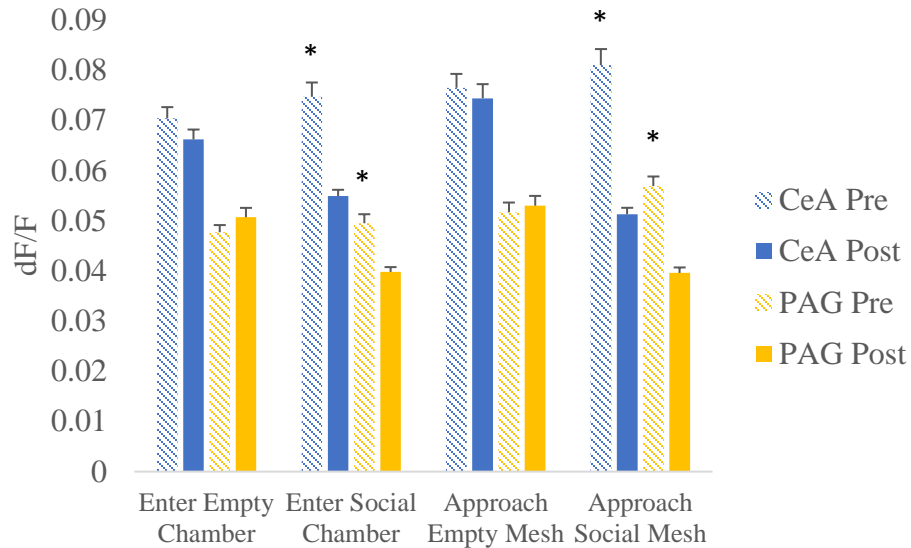
**Figure 9 Photometry Traces Entering Chambers During U-Chamber Social Test** Mean and standard error of photometry recordings of CeA-OTR neurons in the CeA (gray) and vIPAG/LPT (blue) during the U-Chamber Social Test. Recordings are from 6 mice, 39 trials. Pre and Post refer to before and after a stimulus mouse is added the social chamber, respectively. Enter Empty Chamber Pre ( $n_{\text{CeA}} = 160$ ,  $n_{\text{vIPAG}} = 178$ ), and Enter Empty Chamber Post ( $n_{\text{CeA}} = 223$ ,  $n_{\text{vIPAG}} = 235$ ) do not appear to show changes around event. Enter Social Chamber Pre ( $n_{\text{CeA}} = 174$ ,  $n_{\text{vIPAG}} = 151$ ) and Enter Social Chamber Post ( $n_{\text{CeA}} = 418$ ,  $n_{\text{vIPAG}} = 387$ ) show increased activity around event with peaks just after or at event, respectively. vIPAG/LPT recordings are similar, with lower dF/F compared to CeA recordings.



**Figure 10 Photometry Traces Approaching Mesh in U-Chamber Social Test** Mean and standard error of photometry recordings of CeA-OTR neurons in the CeA (gray) and vIPAG/LPT (blue) during the U-Chamber Social Test. Recordings are from 6 mice, 39 trials. Pre and Post refer to before and after a stimulus mouse is added the social chamber, respectively. Approach Empty Mesh Pre ( $n_{\text{CeA}} = 134$ ,  $n_{\text{vIPAG}} = 167$ ) and Approach Social Mesh Pre ( $n_{\text{CeA}} = 168$ ,  $n_{\text{vIPAG}} = 146$ ) show increased activity before and around 10s post event. Approach Empty Mesh Post ( $n_{\text{CeA}} = 150$ ,  $n_{\text{vIPAG}} = 160$ ) shows increased activity around event. Approach Social Mesh Post ( $n_{\text{CeA}} = 313$ ,  $n_{\text{vIPAG}} = 257$ ). vIPAG/LPT recordings are similar, with lower dF/F compared to CeA recordings.



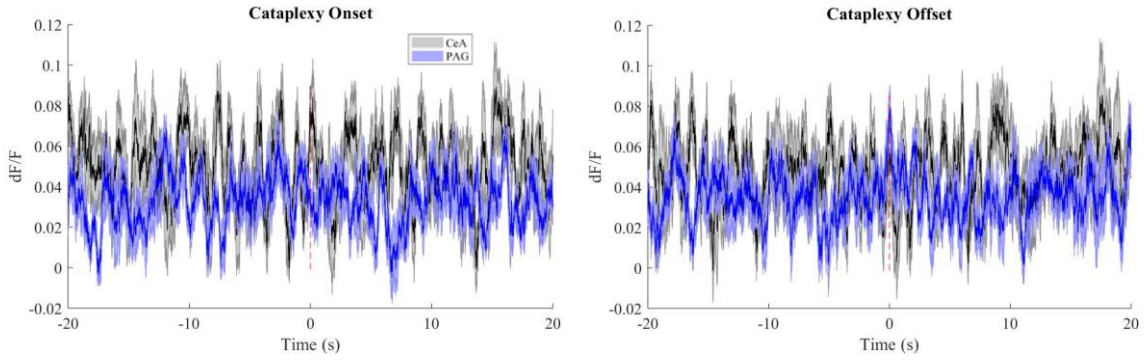
**Figure 11 Activity Before and After Events in the U-Chamber Social Test** Mean and SEM of activity 2 epochs (20s) before and after events in the U-Chamber Social Test of CeA-OTR neurons in the CeA and vIPAG/LPT measured using fiber photometry. Recordings from 6 mice, 39 trials shown. Enter Empty Chamber Pre ( $n_{\text{CeA}} = 160$ ,  $n_{\text{vIPAG}} = 178$ ), Enter Social Chamber Pre ( $n_{\text{CeA}} = 174$ ,  $n_{\text{vIPAG}} = 151$ ), Approach Empty Mesh Pre ( $n_{\text{CeA}} = 134$ ,  $n_{\text{vIPAG}} = 167$ ), Approach Social Mesh Pre ( $n_{\text{CeA}} = 168$ ,  $n_{\text{vIPAG}} = 146$ ), Enter Empty Chamber Post ( $n_{\text{CeA}} = 223$ ,  $n_{\text{vIPAG}} = 235$ ), Enter Social Chamber Post ( $n_{\text{CeA}} = 418$ ,  $n_{\text{vIPAG}} = 387$ ), Approach Empty Mesh Post ( $n_{\text{CeA}} = 150$ ,  $n_{\text{vIPAG}} = 160$ ), Approach Social Mesh Post ( $n_{\text{CeA}} = 313$ ,  $n_{\text{vIPAG}} = 257$ ). Significantly decreased ( $p < 0.05$ ) activity after entering Empty Chamber Pre was measured in the vIPAG/LPT. A significant increase in activity after entering Social Chamber Pre was measured in the CeA. Otherwise, no significant differences before and after events were measured.



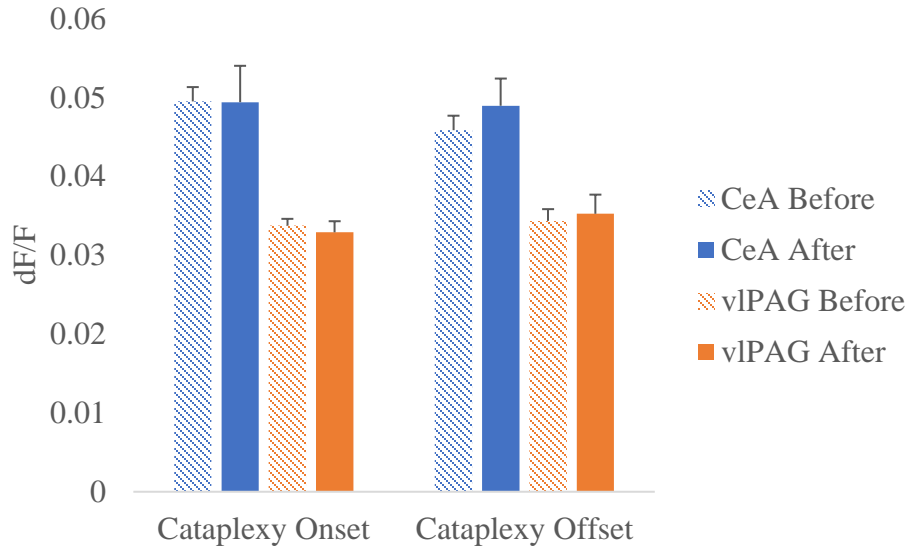
**Figure 12 Maximum Activity Near Events in U-Chamber Social Test** Mean and SEM shown for maximum activity of CeA-OTR neurons in the CeA and vIPAG/LPT [-1,5] seconds around events Pre (before stimulus mouse added to chamber) and Post (after stimulus mouse added to chamber). Recordings taken from 6 mice, 39 trials. Enter Empty Chamber Pre ( $n_{\text{CeA}} = 160$ ,  $n_{\text{vIPAG}} = 178$ ), Enter Social Chamber Pre ( $n_{\text{CeA}} = 174$ ,  $n_{\text{vIPAG}} = 151$ ), Approach Empty Mesh Pre ( $n_{\text{CeA}} = 134$ ,  $n_{\text{vIPAG}} = 167$ ), Approach Social Mesh Pre ( $n_{\text{CeA}} = 168$ ,  $n_{\text{vIPAG}} = 146$ ), Enter Empty Chamber Post ( $n_{\text{CeA}} = 223$ ,  $n_{\text{vIPAG}} = 235$ ), Enter Social Chamber Post ( $n_{\text{CeA}} = 418$ ,  $n_{\text{vIPAG}} = 387$ ), Approach Empty Mesh Post ( $n_{\text{CeA}} = 150$ ,  $n_{\text{vIPAG}} = 160$ ), Approach Social Mesh Post ( $n_{\text{CeA}} = 313$ ,  $n_{\text{vIPAG}} = 257$ ). No significant difference Pre and Post observed for Empty Chamber or Empty Mesh. Significantly decreased ( $p < 0.05$ ) maximum activity Post compared to Pre for Social Chamber and Social Mesh in the CeA and vIPAG/LPT.

Cataplexy occurred 6 times during the U-Chamber Social Test. While there was an empty chamber designated non-social zone, cataplexy that occurred there was within 5 min of social interaction, our definition of social cataplexy based on mean time to first cataplexy from data from Social Reunification Tests. In addition, one episode of cataplexy occurred before a stimulus mouse was added which is excluded from analysis. Both at cataplexy offset and onset, peaks in CeA activity occurred at event with similar peaks occurring several times before and after transitions (Figure 13). vIPAG/LPT traces showed increased activity ( $\sim 0.06$  dF/F) prior to onset ( $-3s$ ), and increased activity at

offset ( $\sim 0.06$  dF/F) (Figure 13). No significant changes in mean activity around transitions (Figure 14).



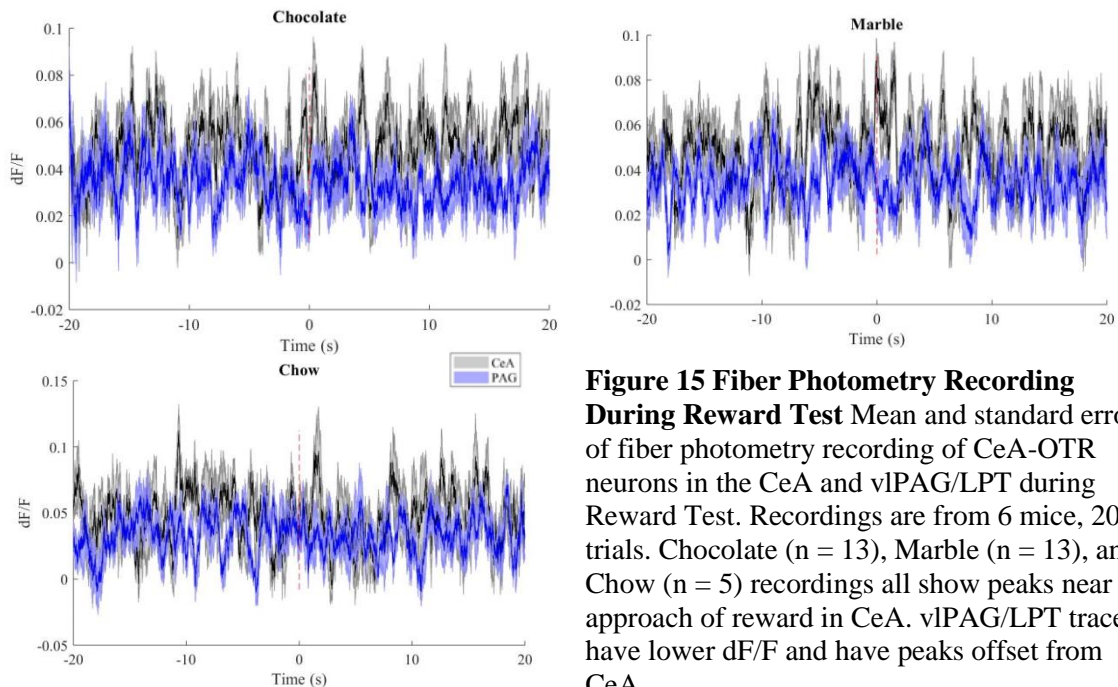
**Figure 13 Fiber Photometry Recording Cataplexy During U-Chamber Social Test** Mean and standard error of fiber photometry recording of CeA-OTR neurons in the CeA and vPAG/LPT during U-Chamber Social Test. Recordings are from 6 mice, 39 trials. Cataplexy ( $n = 6$ ) only occurred in presence of stimulus mouse within 5 minutes of social interaction. Both Cataplexy Onset and Offset show peaks that coincide with  $t = 0s$  in CeA. Similar peaks are otherwise present. vPAG/LPT trace shows increased activity prior to onset, and increased activity at offset.

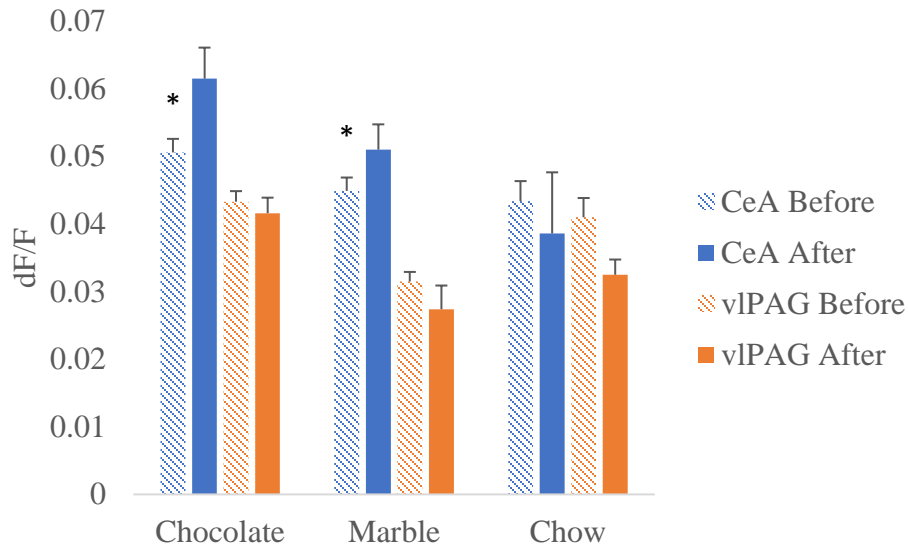


**Figure 14 Activity Around Cataplexy Onset and Offset During U-Chamber Social Test** Mean and SEM of photometry recording of CeA-OTR neurons in CeA and vPAG/LPT during U-Chamber Social Test shown for 2 epochs (20s) before and after cataplexy onset and cataplexy offset. Recordings are from 6 mice, 39 trials. Recordings do not show significant change in mean activity around cataplexy-wake state changes ( $n = 6$ ).

## ***Reward Test***

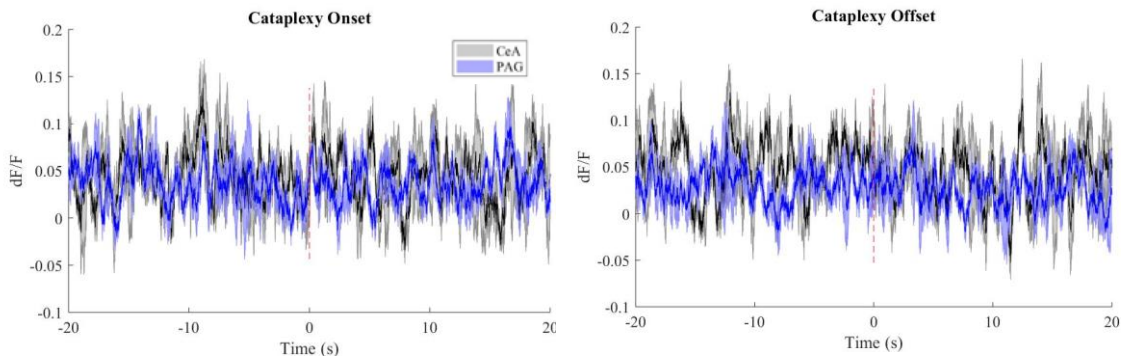
The Reward Test was conducted within the home cage like the Home Cage Social Test, and activity of CeA-OTR neurons in the CeA and vPAG/LPT was measured at low excitation light power using fiber photometry around approach to one of three reward items: chocolate, marble, and chow. There are peaks in activity just after an approach to all three items in the CeA and vPAG/LPT (Figure 15). An approach to a marble and chocolate showed increased activity about 5s before event in the CeA and vPAG/LPT, 10s before for chow (Figure 15). Mean activity was significantly increased after an approach to chocolate and marble in the CeA (Figure 16).



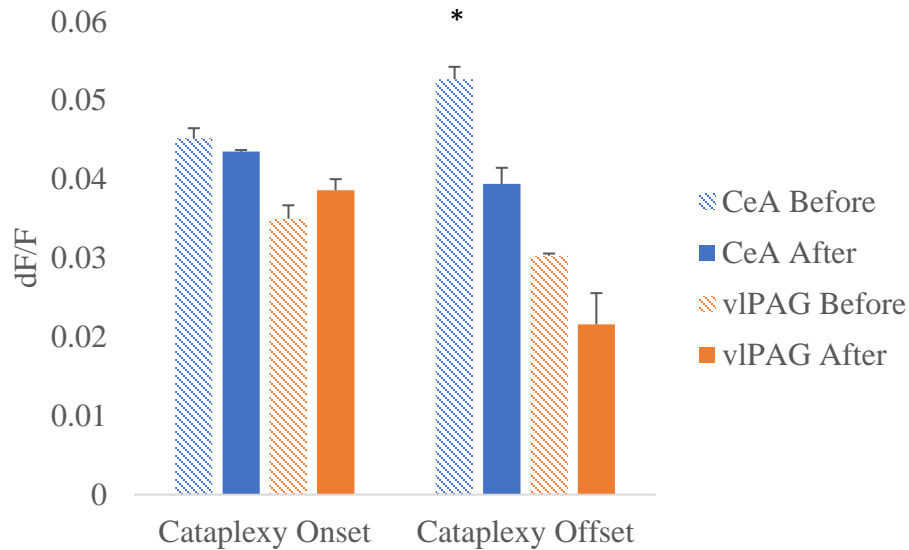


**Figure 16 Activity Around Approach to Reward** Mean and SEM of photometry recording of CeA-OTR neurons in CeA and vIPAG/LPT for 2 epochs (20s) before and after approach to chocolate (n = 13), marble (n = 13), and chow (n = 5). Recordings are from 6 mice, 20 trials. Significant increases ( $p < 0.05$ ) in mean dF/F measured after approach to chocolate and marble in the CeA.

Cataplexy, during the Reward Test, occurred 3 times. Photometry recordings show increased activity ( $\sim 0.1$  dF/F in the CeA,  $\sim 0.06$  dF/F in the vIPAG/LPT) at cataplexy onset, with increased activity ( $\sim 0.13$  dF/F) about 10s before onset in the CeA (Figure 17). Cataplexy offset showed some increased activity around offset ( $\sim 0.1$  dF/F), with a significant decrease in mean activity after offset in the CeA (Figure 17, Figure 18).



**Figure 17 Fiber Photometry Recordings of Cataplexy During Reward Test** Mean and standard error of photometry recordings of CeA-OTR neurons in the CeA and vIPAG/LPT during the Reward Test. Recordings are from 6 mice, 20 trials. Cataplexy (n = 3) traces show increased activity in the CeA at onset, and generally lower, almost constant, dF/F in vIPAG/LPT.



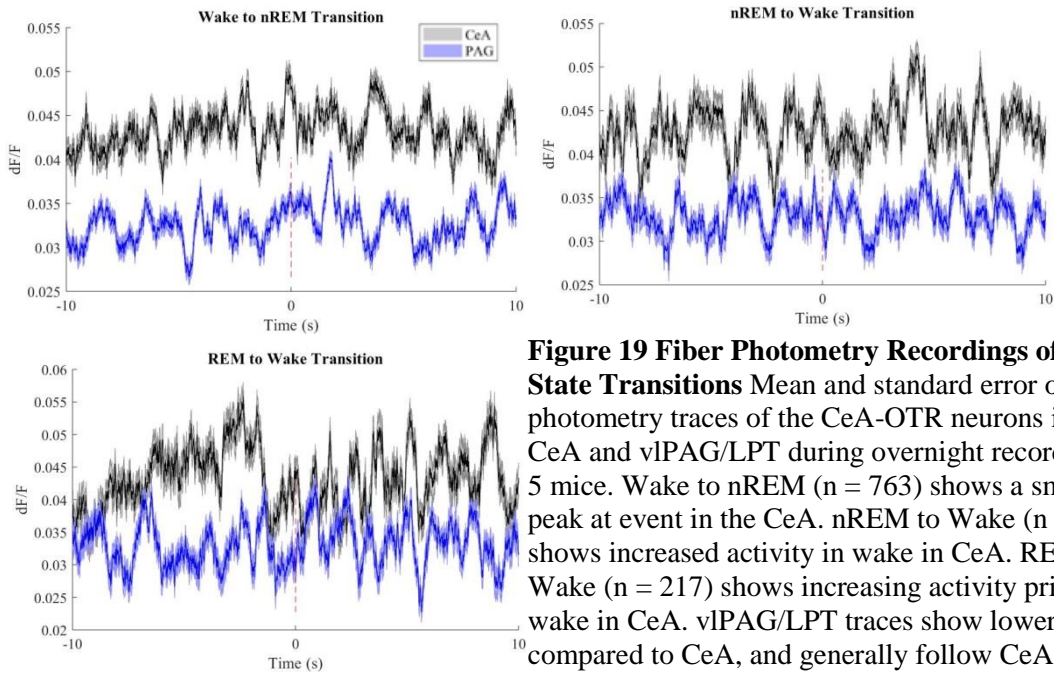
**Figure 18 Activity Around Cataplexy Onset and Offset During Reward Test** Mean and SEM of photometry recordings of CeA-OTR neurons in CeA and vIPAG/LPT shown for 2 epochs (20s) before and after cataplexy ( $n = 3$ ) onset and offset during the Reward Test. Recordings are from 6 mice, 20 trials. Significantly decreased ( $p < 0.05$ ) mean activity in CeA after cataplexy offset.

### *State Transitions*

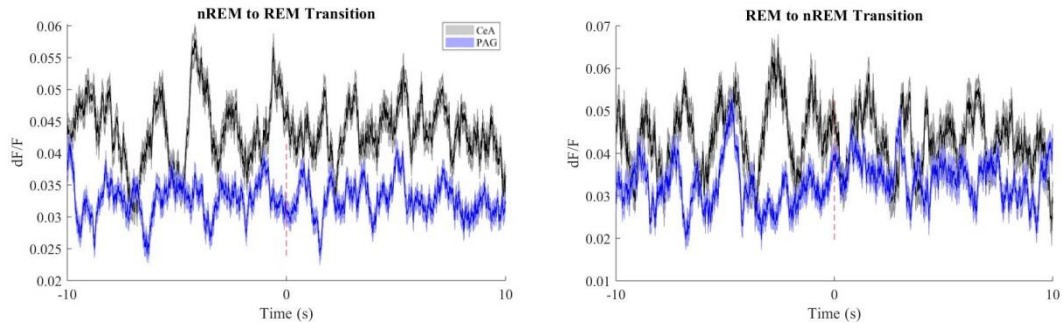
Photometry recordings of CeA-OTR neurons in the CeA and vIPAG/LPT for analyzing state transitions were taken from 12-hour overnight recordings of the mice. These recordings were taken at low LED output power (0.1nW), with some complications from variation in power at the vIPAG/LPT LED. Wake to nREM transition showed a small increase in a few seconds leading up to transition followed by a small decrease (Figure 19). nREM to wake showed increased activity (0.05 dF/F) about +5s in the CeA (Figure 19). REM to Wake showed increasing activity in REM up to about -2s in CeA (Figure 19). nREM to REM transitions showed higher amplitude activity before transition in the CeA (Figure 20). REM to nREM transition showed high activity 2s before transition to nREM in CeA (Figure 20). vIPAG/LPT traces appear to parallel CeA

traces with smaller changes in activity (Figure 19, Figure 20). Pearson correlation coefficient between CeA and vIPAG/LPT ( $r = 0.0007$ ) taken from five overnight recordings indicated almost no correlation between CeA and vIPAG/LPT traces.

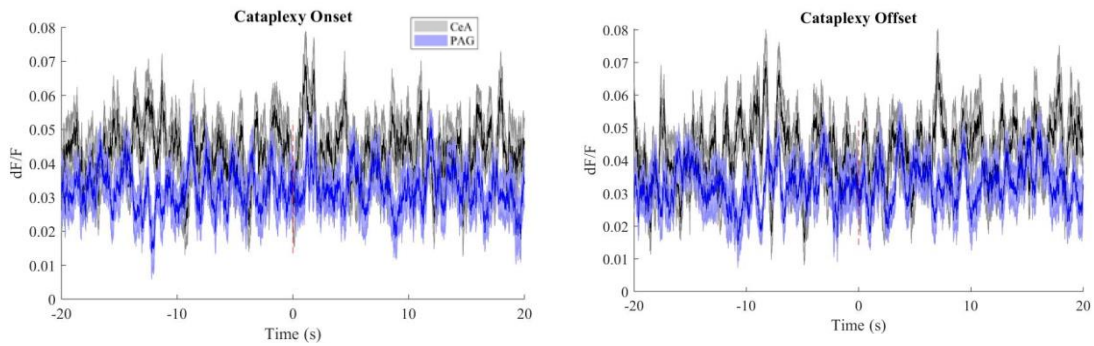
Cataplexy occurred 77 times during overnight recordings. Maximum activity in the CeA at onset was  $\sim 0.07$  dF/F, as well as increased activity in vIPAG/LPT at onset (Figure 21). At cataplexy offset increased activity was observed at  $\pm 8$ s ( $\sim 0.07$  dF/F) (Figure 21). Mean activity across behavioral states was significantly decreased during cataplexy compared REM, nREM, and wake in both CeA and vIPAG/LPT (Figure 22).



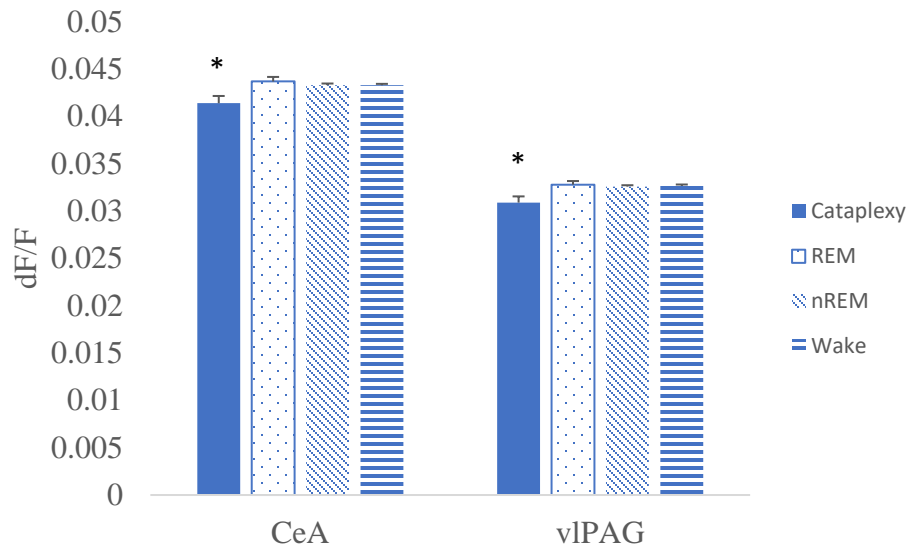
**Figure 19 Fiber Photometry Recordings of Wake State Transitions** Mean and standard error of photometry traces of the CeA-OTR neurons in the CeA and vIPAG/LPT during overnight recordings of 5 mice. Wake to nREM ( $n = 763$ ) shows a small peak at event in the CeA. nREM to Wake ( $n = 418$ ) shows increased activity in wake in CeA. REM to Wake ( $n = 217$ ) shows increasing activity prior to wake in CeA. vIPAG/LPT traces show lower dF/F compared to CeA, and generally follow CeA signal.



**Figure 20 Fiber Photometry Recordings of Sleep Transitions** Mean and standard error of photometry recordings of CeA-OTR neurons in the CeA and vIPAG/LPT during overnight recordings of 5 mice. nREM to REM transition ( $n = 299$ ) shows higher activity before transition in the CeA. REM to nREM transition ( $n = 96$ ) shows some increased activity just prior to transition in CeA. vIPAG/LPT signal follows CeA with lower dF/F.



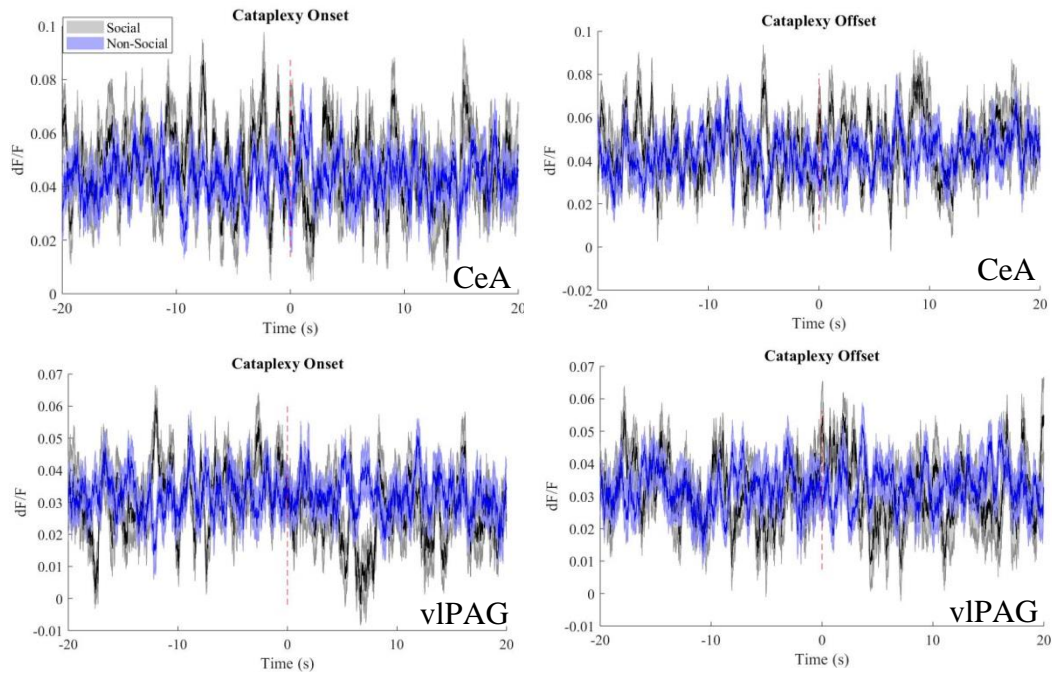
**Figure 21 Fiber Photometry Recordings of Cataplexy** Mean and standard error of photometry recordings of CeA-OTR neurons in CeA and vIPAG/LPT around cataplexy transitions ( $n = 77$ ) during overnight recordings of 5 mice. Cataplexy Onset shows increased activity at onset in CeA. Cataplexy Offset does not show significant activity change near offset, some increased activity an epoch before and after offset in CeA. vIPAG/LPT traces follow CeA and have lower dF/F.



**Figure 22 Activity of CeA-OTR Neurons Across Arousal States** Mean and SEM of activity of CeA-OTR neurons in CeA and vIPAG/LPT, measured using fiber photometry during overnight recordings of 5 mice, across each behavioral state: Cataplexy (n = 386 epochs), REM (n = 1,154 epochs), nREM (n = 8,302 epochs), Wake (n = 12,426 epochs). CeA-OTR neurons have significantly lower activity ( $p < 0.05$ ) in cataplexy compared to wake, nREM, or REM in both CeA and vIPAG/LPT.

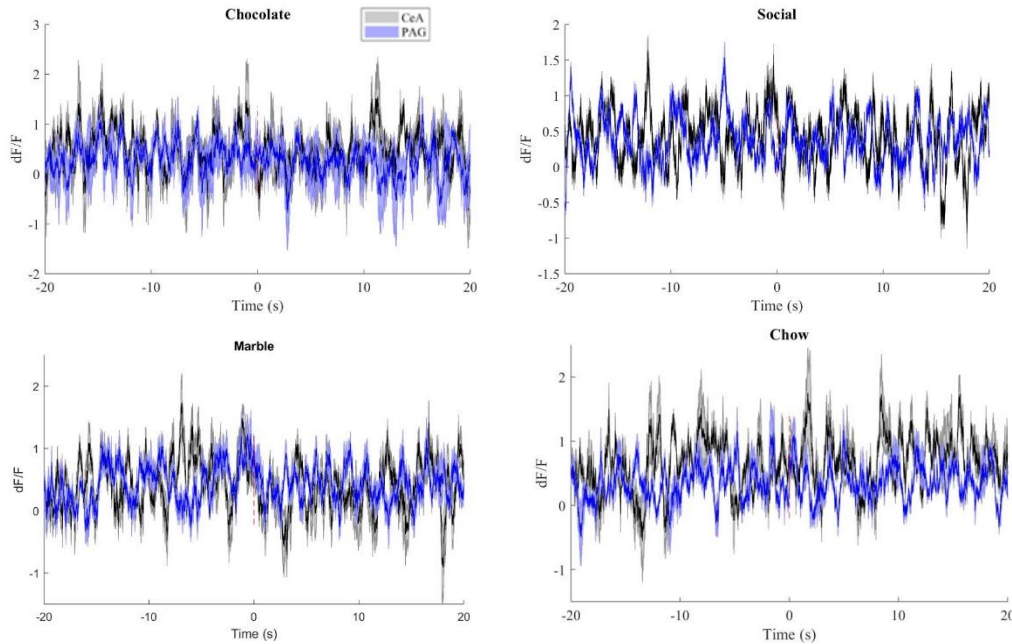
To investigate potential differences between spontaneous cataplexy and social cataplexy, social cataplexy from both the Home Cage Social Test and U-Chamber Social Test was grouped and plotted with spontaneous cataplexy from overnight recordings. In the CeA at onset, we can see a peak in activity at onset and just before onset in both social and non-social with social having a maximum preceding event ( $-3s$ ), and non-social with a maximum at event. Similar peaks were present in vIPAG/LPT onset shifted about  $+1s$  with a pronounced decrease in activity around  $-7s$  in the social cataplexy recordings (Figure 23). At cataplexy offset, increases were observed in CeA for both social and non-social cataplexy at least  $5s$  before and after with an increase just after offset in both cases (Figure 23). In the vIPAG/LPT, there was increased activity at offset in both social and non-social cataplexy (Figure 23). In all traces, there was noticeable

peak and standard error overlap, with social cataplexy exhibiting more high amplitude activity compared to non-social cataplexy recordings (Figure 23).



**Figure 23 Social v. Non-Social Cataplexy** Mean and standard error of fiber photometry traces of CeA-OTR neurons in the CeA and vIPAG/LPT. Recordings are from 8 mice. Social Cataplexy ( $n = 12$ ) is defined as occurring within 5 minutes of social interaction. Social cataplexy is not significantly different from non-social cataplexy ( $n = 77$ ). Although, higher amplitude activity is observed in CeA and vIPAG/LPT in presence of social stimuli, with peaks at cataplexy onset, and more pronounced increase in activity post-offset. Pearson correlation coefficients are as follows: Cataplexy Onset CeA ( $r = -0.099$ ), Cataplexy Onset vIPAG/LPT ( $r = -0.0775$ ), Cataplexy Offset CeA ( $r = 0.0608$ ), Cataplexy Offset vIPAG/LPT ( $r = 0.0426$ ). In general, very small to no correlation between events.

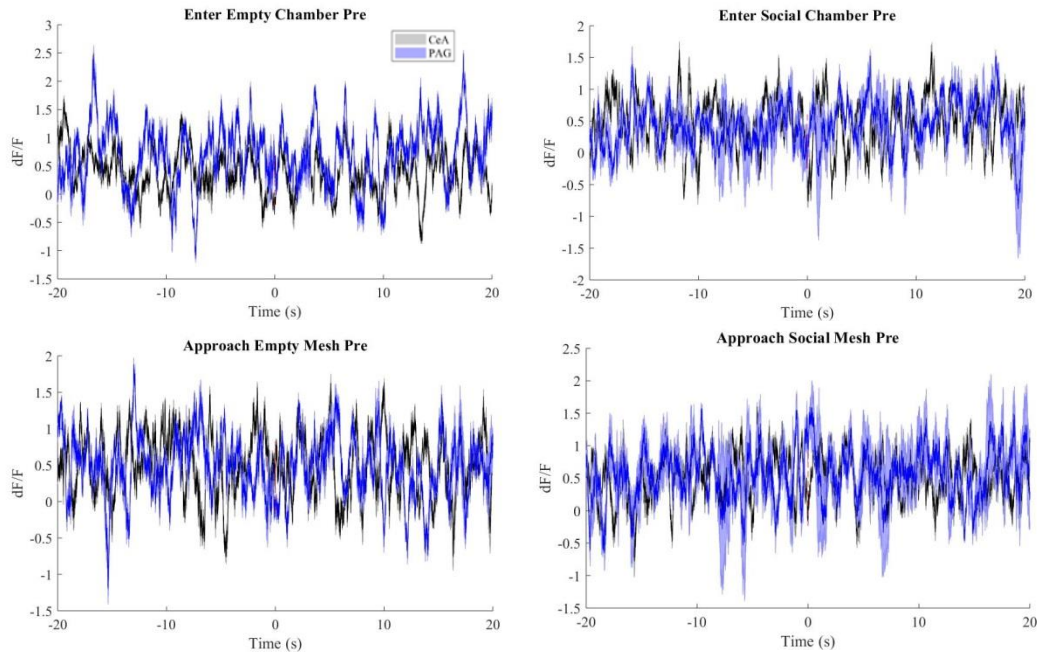
## Higher LED Power Preliminary Results



**Figure 24 Fiber Photometry Recording During Home Cage Tests** Mean and standard error of non-simultaneous photometry recordings taken of CeA-OTR neurons in the CeA (gray) and vIPAG (blue) shown on approach to chocolate ( $n_{\text{CeA}} = 3$ ,  $n_{\text{vIPAG}} = 3$ ), approach to marble ( $n_{\text{CeA}} = 2$ ,  $n_{\text{vIPAG}} = 2$ ), approach to chow ( $n_{\text{CeA}} = 2$ ,  $n_{\text{vIPAG}} = 2$ ), and upon initial social interaction when a stimulus mouse is added to the home cage ( $n_{\text{CeA}} = 12$ ,  $n_{\text{vIPAG}} = 12$ ). Recordings of 2 mice, 3 trials for CeA and vIPAG respectively, done with excitation light at  $0.2\mu\text{W}$ . Recordings show increase in activity up to 3s prior to approach to chocolate, marble and social interaction in both CeA and vIPAG. Activity increases 3s following approach to chow in CeA, and within 1s following approach to chow in vIPAG.

At a higher LED power output of  $0.2\mu\text{W}$ , recordings were done in the home cage exposing the mice to chocolate, marble, chow and to social interaction. This data should be considered preliminary due to small number of events. In these traces, we can see a small increase in activity about 2s before approach to chocolate in the vIPAG ( $\sim 1.1$  dF/F) and in the CeA ( $\sim 2.2$  dF/F) (Figure 24). Activity increased within a few seconds before an approach to marble as well with dF/F about 1.5 in both CeA and vIPAG (Figure 24). Recordings of approach to chow showed increase in the vIPAG 2s before and at event ( $\sim 1.3$  dF/F) with increase in CeA at 2s following event ( $\sim 2.2$  dF/F) (Figure 24). At  $-2$ s to

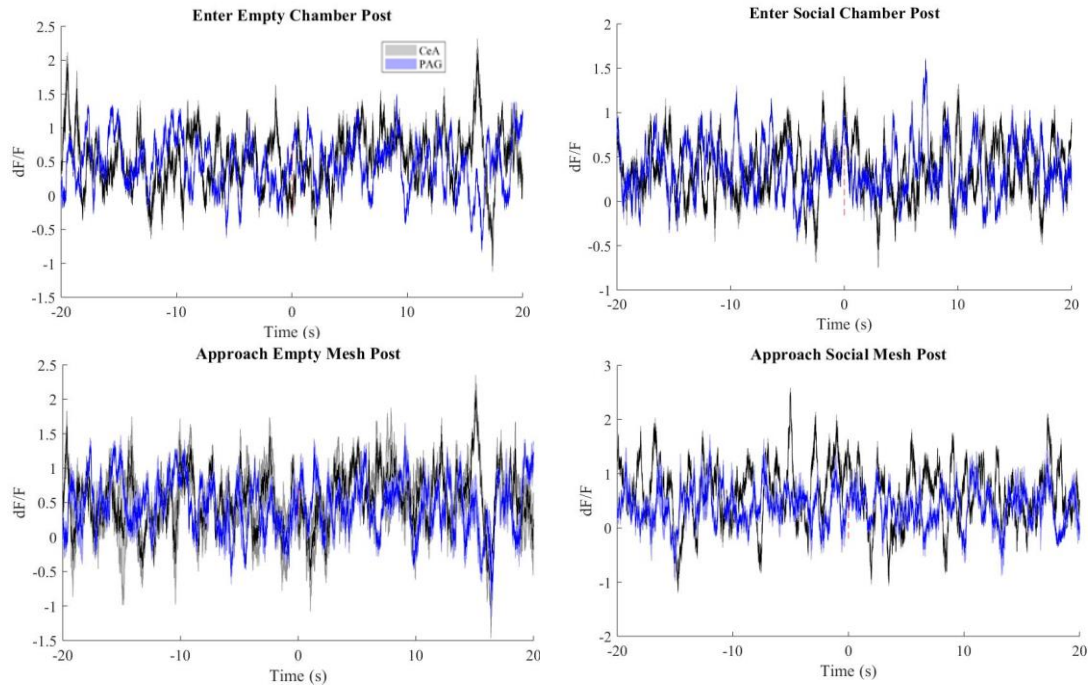
social interaction activity reached about 1.6 dF/F in the CeA, similar activity occurred in the vIPAG traces at -5s (Figure 24).



**Figure 25 Fiber Photometry Recordings at Higher LED Power During U-Chamber Social Test Pre** Mean and standard error of non-simultaneous fiber photometry recordings of CeA-OTR neurons taken from CeA (gray) and vIPAG (blue) at  $0.2\mu\text{W}$  LED power output. Recordings are from 2 mice, 5 trials all taken before the stimulus was added to the test chamber (Pre). Enter Empty Chamber Pre ( $n_{\text{CeA}} = 11$ ,  $n_{\text{vIPAG}} = 8$ ), Enter Social Chamber Pre ( $n_{\text{CeA}} = 13$ ,  $n_{\text{vIPAG}} = 6$ ), Approach Empty Mesh Pre ( $n_{\text{CeA}} = 12$ ,  $n_{\text{vIPAG}} = 7$ ), Approach Social Mesh Pre ( $n_{\text{CeA}} = 16$ ,  $n_{\text{vIPAG}} = 5$ ). Recordings before stimulus mouse was added to the test chambers show peak at event with similar peaks throughout recording.

Mice were tested in the U-Chamber Social Test with higher excitation light power ( $0.2\mu\text{W}$ ). Entering the empty chamber before a stimulus mouse was added to the U-Chamber showed increased activity at 1s in the CeA ( $\sim 0.75$  dF/F) and in the vIPAG ( $\sim 0.75$  dF/F) with maximum activity in the vIPAG present more than an epoch before and after event ( $\sim 2.5$  dF/F) (Figure 25). Entering the social chamber pre showed perhaps a slight increase in CeA and vIPAG a couple seconds before event but no dramatic trend (Figure 25). Approaching the empty mesh and social mesh pre, recordings showed

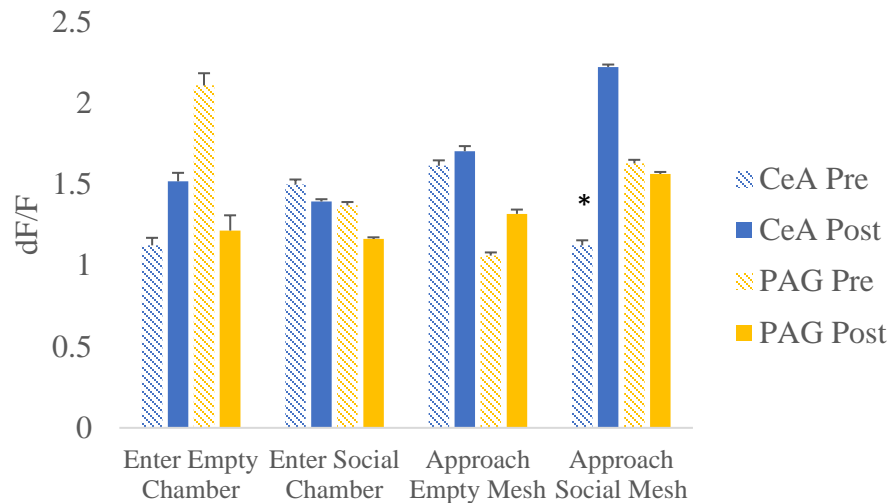
activity at 1.5 dF/F CeA and vIPAG, respectively, near event, with similar activity occurring several times within an epoch of event (Figure 25).



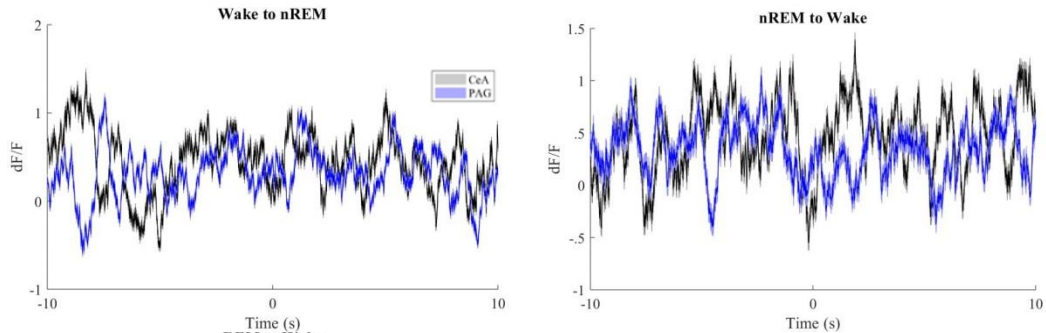
**Figure 26 Fiber Photometry Recordings at Higher LED Power During U-Chamber Social Test Post** Mean and standard error of non-simultaneous fiber photometry recordings of CeA-OTR neurons taken from CeA (gray) and vIPAG (blue) at  $0.2\mu\text{W}$  LED power output. Recordings are from 2 mice, 5 trials for CeA and vIPAG respectively, all taken after the stimulus was added to the test chamber (Post). Enter Empty Chamber Post ( $n_{\text{CeA}} = 11$ ,  $n_{\text{vIPAG}} = 13$ ), Enter Social Chamber Post ( $n_{\text{CeA}} = 16$ ,  $n_{\text{vIPAG}} = 15$ ), Approach Empty Mesh Post ( $n_{\text{CeA}} = 6$ ,  $n_{\text{vIPAG}} = 5$ ), Approach Social Mesh Post ( $n_{\text{CeA}} = 15$ ,  $n_{\text{vIPAG}} = 9$ ). Recordings show increased activity in CeA before entering empty chamber post and before entering social chamber post. Recordings show increased activity up to 5s before event with maximum activity about 5s before approach to social mesh.

After the stimulus mouse was added to the chamber, we saw increased activity 1s before entering the empty chamber post with similar peaks near 1.5 dF/F throughout the trace in both the CeA and vIPAG (Figure 26). Entering the social chamber post traces showed increased activity about 2s before and at event in the CeA and vIPAG ( $\sim 1.3$  dF/F) with maximum in the vIPAG at 8s (1.5 dF/F) (Figure 26). Approaching the empty mesh post showed activity at 1.5 dF/F a few seconds before event in the CeA and at 1 dF/F in

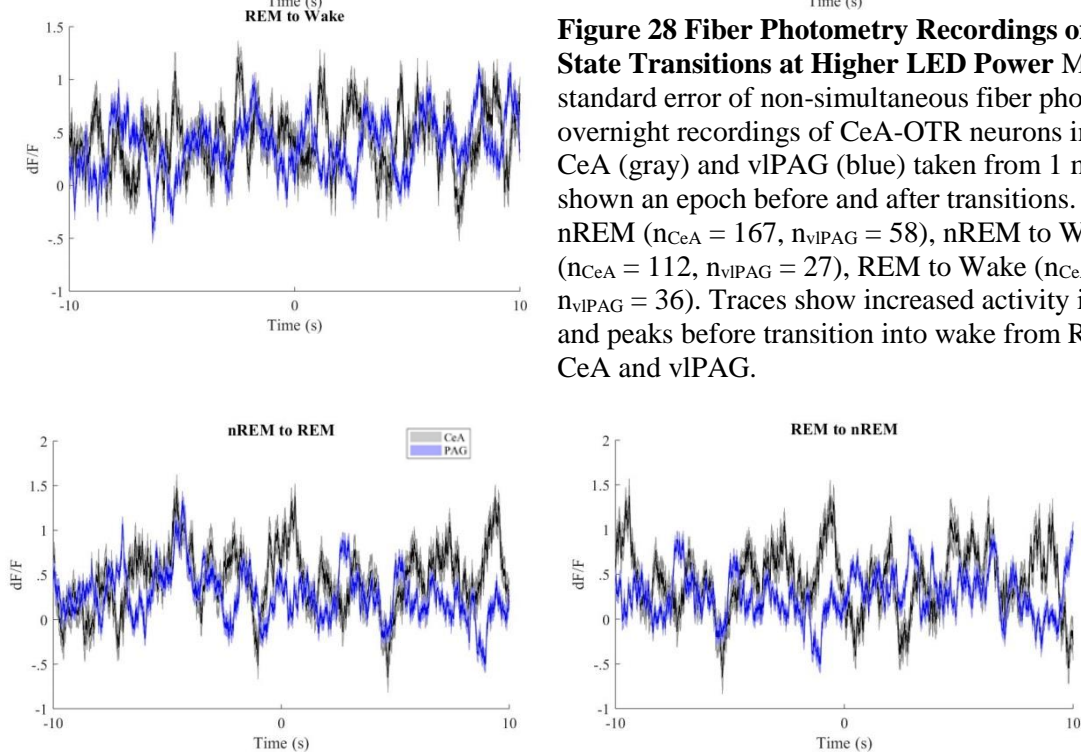
the vIPAG with similar activity throughout the recording (Figure 26). Recordings from the CeA on approach to the social mesh post showed an increase up to 5s before event in CeA (~2.3 dF/F). This peak in CeA upon approach to social mesh was significantly higher than maximum activity near approach to social mesh pre (Figure 27). vIPAG traces showed increased activity at -1s (1.3 dF/F) with similar activity at several places across the trace (Figure 26).



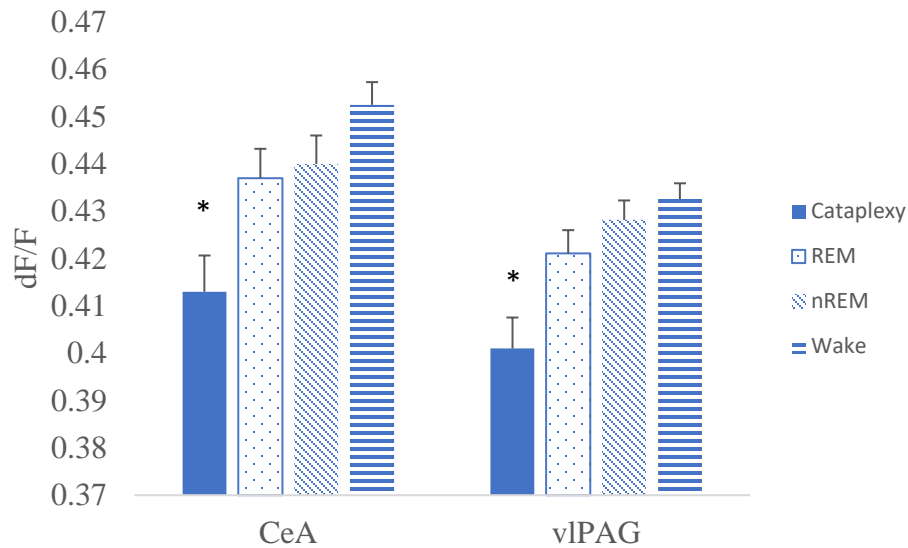
**Figure 27 Maximum Activity Near Events in U-Chamber Social Test at Higher LED Power** Mean and standard error of maximum activity within 3s of event for events in U-Chamber Social Test both before (pre) and after (post) stimulus mouse present in test chamber. Recordings were taken from CeA and vIPAG, 5 trials each in 2 mice. Enter Empty Chamber Pre ( $n_{\text{CeA}} = 11$ ,  $n_{\text{vIPAG}} = 8$ ) Enter Empty Chamber Post ( $n_{\text{CeA}} = 11$ ,  $n_{\text{vIPAG}} = 13$ ), Enter Social Chamber Pre ( $n_{\text{CeA}} = 13$ ,  $n_{\text{vIPAG}} = 6$ ), Enter Social Chamber Post ( $n_{\text{CeA}} = 16$ ,  $n_{\text{vIPAG}} = 15$ ), Approach Empty Mesh Pre ( $n_{\text{CeA}} = 12$ ,  $n_{\text{vIPAG}} = 7$ ), Approach Empty Mesh Post ( $n_{\text{CeA}} = 6$ ,  $n_{\text{vIPAG}} = 5$ ), Approach Social Mesh Pre ( $n_{\text{CeA}} = 16$ ,  $n_{\text{vIPAG}} = 5$ ), Approach Social Mesh Post ( $n_{\text{CeA}} = 15$ ,  $n_{\text{vIPAG}} = 9$ ). Maximum activity near approach to social mesh is significantly higher post compared to pre stimulus mouse presence in test chamber ( $p < 0.05$ ).



**Figure 28 Fiber Photometry Recordings of Wake State Transitions at Higher LED Power** Mean and standard error of non-simultaneous fiber photometry overnight recordings of CeA-OTR neurons in the CeA (gray) and vIPAG (blue) taken from 1 mouse shown an epoch before and after transitions. Wake to nREM ( $n_{\text{CeA}} = 167$ ,  $n_{\text{vIPAG}} = 58$ ), nREM to Wake ( $n_{\text{CeA}} = 112$ ,  $n_{\text{vIPAG}} = 27$ ), REM to Wake ( $n_{\text{CeA}} = 43$ ,  $n_{\text{vIPAG}} = 36$ ). Traces show increased activity in wake and peaks before transition into wake from REM in CeA and vIPAG.



**Figure 29 Fiber Photometry Recording of Sleep State Transitions at Higher LED Power** Mean and standard error of non-simultaneous fiber photometry recordings of CeA-OTR neurons in the CeA (gray) and vIPAG (blue) an epoch before and after sleep state transitions, nREM to REM ( $n_{\text{CeA}} = 58$ ,  $n_{\text{vIPAG}} = 65$ ), REM to nREM ( $n_{\text{CeA}} = 16$ ,  $n_{\text{vIPAG}} = 48$ ). Recordings were from one mouse, 10 hours overnight. Increased activity upon transitions into and out of REM in CeA.

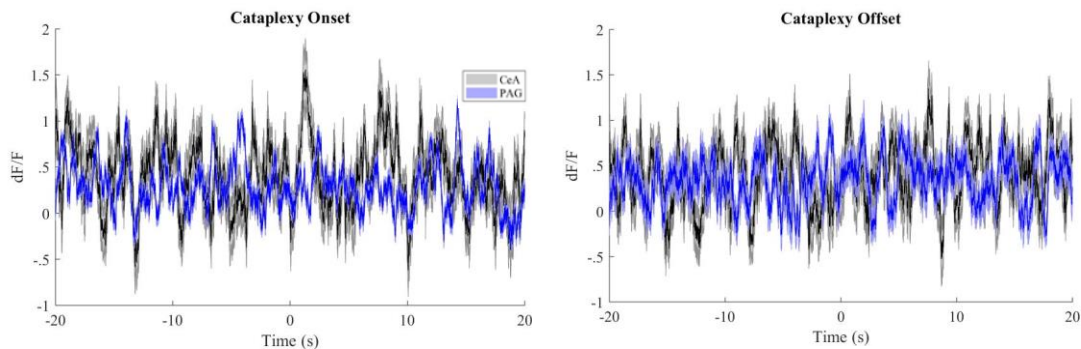


**Figure 30 Activity Across Arousal States at Higher LED Power** Mean and standard error of activity taken from fiber photometry recording of CeA-OTR neurons in CeA and vIPAG from 1 mouse, 10 hours overnight. Cataplexy ( $n_{\text{CeA}} = 76$ ,  $n_{\text{vIPAG}} = 88$ ), REM ( $n_{\text{CeA}} = 217$ ,  $n_{\text{vIPAG}} = 178$ ), nREM ( $n_{\text{CeA}} = 1387$ ,  $n_{\text{vIPAG}} = 1647$ ), Wake ( $n_{\text{CeA}} = 2970$ ,  $n_{\text{vIPAG}} = 2329$ ). Activity is significantly lower during cataplexy compared to nREM, REM, and Wake ( $p < 0.05$ ).

Sleep and wake state transitions were taken from an overnight recording. vIPAG and CeA were recorded separately at  $0.2\mu\text{W}$  excitation light power output. Transition from wake to nREM showed a small increase near event with similar activity throughout recordings apart from higher activity in CeA  $>5\text{s}$  before transition into nREM (Figure 30). Recordings of the transition from nREM to wake showed increased activity in CeA and vIPAG from  $-5\text{s}$  to  $3\text{s}$  with maximum activity in CeA at  $3\text{s}$  near  $1.5\text{ dF/F}$  (Figure 30). Increased activity presented just before REM transitions into nREM in the CeA ( $\sim 1.5\text{ dF/F}$ ) and into wake in the CeA ( $\sim 1.3\text{ dF/F}$ ) and vIPAG ( $\sim 1\text{ dF/F}$ ) (Figure 28, Figure 29). Increased activity occurred  $5\text{s}$  before transition from nREM to REM in the CeA and vIPAG ( $\sim 1.4\text{ dF/F}$ ) (Figure 29). Transitioning from nREM to REM recordings also showed CeA activity near  $1.5\text{ dF/F}$  just following transition (Figure 29). Pearson

correlation coefficient between the CeA and vIPAG taken from one overnight recording ( $r = 0.0019$ ) showed low to no correlation between traces, although correlation was more than double the correlation between CeA and vIPAG traces at low LED power ( $r = 0.0007$ ).

Photometry recording of CeA-OTR neurons at cataplexy onset showed increased activity 1s following transition in CeA ( $\sim 2$  dF/F) with similar peak at 8s (Figure 31). Increased activity in vIPAG occurred at  $-5$ s ( $1.2$  dF/F) (Figure 31). Cataplexy offset traces did not show a clear trend with some increased activity following offset in CeA ( $\sim 1.5$  dF/F) and vIPAG ( $\sim 1$  dF/F) with similar peaks throughout trace. Across states, activity was significantly lower during cataplexy (Figure 30).



**Figure 31 Fiber Photometry Recording of Cataplexy at Higher LED Power** Mean and standard error of non-simultaneous fiber photometry recordings of CeA-OTR neurons in the CeA (gray) and vIPAG (blue). Recordings were from one mouse, 10 hours overnight. Cataplexy onset ( $n_{\text{CeA}} = 9$ ,  $n_{\text{vIPAG}} = 16$ ) shows some increase activity near onset in CeA. Cataplexy offset ( $n_{\text{CeA}} = 9$ ,  $n_{\text{vIPAG}} = 16$ ) shows small increase in activity following offset.

LED Power	Region	Enter Empty Chamber	Enter Social Chamber	Approach Empty Mesh	Approach Social Mesh
0.1nW	CeA	0	–	0	–
	vIPAG	0	–	0	–
0.2 $\mu$ W	CeA	0	0	0	+
	vIPAG	0	0	0	0

**Table 1 Summary of Social Test** 0 indicates no significant trend, + indicates increased activity in presence of stimulus mouse, – indicates decreased activity in presence of stimulus mouse.

LED Power	Region	Chocolate	Marble	Chow
0.1nW	CeA	+	+	0
	vIPAG	0	0	0
0.2 $\mu$ W	CeA	+	+	+
	vIPAG	0	+	+

**Table 2 Summary of Reward Test** 0 indicates no trend, + indicates increased activity.

LED Power	Region	Cat Onset Social	Cat Offset Social	Cat Onset	Cat Offset	During Cataplexy
0.1nW	CeA	0	0	+	0	–
	vIPAG	0	0	0	0	–
0.2 $\mu$ W	CeA	n/a	n/a	+	0	–
	vIPAG	n/a	n/a	0	0	–

**Table 3 Summary of Cataplexy Transitions** 0 indicates no response, + indicates response with increased activity near event, – indicates decreased activity in comparison to all other arousal states.

LED Power	Wake to nREM	REM to Wake	nREM to Wake	nREM to REM	REM to nREM
0.1nW	0	+	0	+	+
	0	0	0	0	0
0.2 $\mu$ W	0	+	0	+	+
	0	+	0	0	0

**Table 4 Summary of Sleep Transitions** 0 indicates no response, + indicates response with increased activity near event

## DISCUSSION

The experiments described were designed to characterize the activity pattern of the CeA-OTR neurons and their terminals in the vIPAG/LPT across different arousal states and in response to different reward stimuli. We investigated changes in activity of these neurons during social cataplexy and spontaneous cataplexy to determine if these neurons are involved with initiation or maintenance of cataplexy.

### *Social and Reward Tests*

Under social conditions, in this case both the Home Cage Test and the U-Chamber Test we could measure increased activity in response to the events. The Home Cage Test yielded high variability in response to social interaction including some trials with a decrease following an approach by the stimulus mouse, and some trials with an increase before the event with a mutual interaction or increases following an approach of the stimulus mouse. This variability of response makes it difficult to draw conclusions regarding general approach to social interaction, which makes sense given the complex nature of interactions in this setting that we are trying to measure. For example, a mutual interaction comprises every interaction where it is not clear which mouse approached the other first so high variability is expected because this potentially includes several kinds of interactions. The same is true for either approach to stimulus or approach by stimulus. In fact, approach by stimulus can also include following behavior which has negative associations for the mice, when positive interactions are more likely to be triggers of cataplexy. A benefit of the U-Chamber Social Test is that it limits social interactions by allowing free movement only to the subject mouse.

The U-Chamber Social Test had more consistent responses of CeA-OTR neurons with increases near events and no dramatic changes in mean activity before and after events like with an approach by the stimulus mouse in the Home Cage Social Test. Analysis of maximums at and just after events Pre and Post showed no changes in pre and post for the empty chamber which is expected because that should be theoretically the same event, with a decrease Post compared to Pre for the social chamber. When we compare these photometry recordings, we can see many peaks more than 1s before the event, any peak prior to the event would not be included in this mean. There are peaks of fluorescence about 3 seconds before events so there could be an anticipatory effect where the cells respond prior to social interaction most likely in the decision to enter the chamber or the decision to approach the mesh (DiFeliceantonio and Berridge 2012). Higher activity before an event would result in a higher baseline used to calculate  $dF/F$ . Therefore,  $dF/F$  would be smaller which may explain smaller  $dF/F$  observed at approach to social mesh Post compared to Pre.

In the social interaction tests, it is difficult to define the onset of an interaction, and thus alignment of signals can be a challenge. In these tests, we used direct contact, or the exact time the mouse's nose enters a chamber as  $t = 0s$ . The "exact" time can vary by  $\pm 1s$  because that is the precision of Sleep Sign. In addition, mice move at different speeds thereby the time to enter the chambers differs across trials, and perhaps deciding on an action at different times would present with responses in the CeA-OTR neurons at different relative times to  $t = 0s$ , peaks which would become smaller when we take a mean. While trends presented here represent what we see in individual traces, the relative

change may be smaller than what occurs in the neurons. In addition, while there is some error at  $t = 0s$ , we may not be able to confidently conclude that there is an increase in activity at, just before, or just after an event, we can be certain that there is a response to social interaction in these cells as evidenced by increased activity entering the social chamber post and approaching the social mesh post.

This same challenge in temporal alignment applies to the reward test as at approach to each reward we see similar increases in activity, but with the same error of start cannot conclude precise timing of response. Benefits of the reward test are that only one kind of event can happen, and that although the mice are freely moving, they tend to approach the rewards only once so we can be sure the mouse is not doing anything significant before or after the approach and interaction with the reward item.

To achieve a true baseline with social tests, that is that the mouse is not doing anything 20s before event, and anything following an event could only be from the event, at its most complex, one could move further in the direction of the U-Chamber Test compared to the Home Cage Social Test. That means a further reduction in confounding variables surrounding social interaction. This can be accomplished by moving away from the natural and complex social interactions observed in the Home Cage Social Test, toward restriction of free motion of both the stimulus mouse, and the subject mouse, with implementation of the cost-benefit test by the Graybiel lab at McGovern Institute of Brain Research. This includes a T-maze with trained and habituated mice (Friedman et al. 2015). Basically, training the subject mice to make decisions and then go either all way to the social mesh or all the way to the empty mesh, and use a moving barrier to then

keep the mice in the respective chamber for 20s. All trials would begin with the mouse in a neutral space at the end of the maze. This would allow one to restrict events around an approach of a mesh and have clear baseline before they are free to decide and enter either chamber. This maze could also be used for reward tests which could potentially increase the number of events. A much simpler way would be to do the Home Cage Social Test and add and remove the mouse more frequently analyzing only the first interaction.

Using a T-maze and trained mice may improve measuring CeA-OTR neuron activity in response to social events but not so much for social cataplexy. A benefit of the Home Cage Social Test is that it is conducted in a space the mouse is comfortable in possibly resulting in higher rates of cataplexy, about one per trial on average, as opposed to the U-Chamber Social Test where we see about 2 in every 10 trials. While a T-maze test like this would require lots of training and habituation to the maze, it would still never be as comfortable for the mice as their home cages, possibly resulting in lower rates of cataplexy as we see with the U-Chamber Social Test.

### *State Transitions*

In addition to social and rewarding stimuli, we looked at state transitions. Activity during wakefulness around transitions could just be responses to other things in the wake state. When looking at transitions, peaks that occur at  $t = 0$ s are like other peaks in the traces. Except for peaks preceding transition out of REM into Wake or nREM. Even with some error in determining  $t = 0$ s, those peaks are either just before the transition or a few seconds before the transition. Since the mouse is asleep, these responses cannot be attributed to external stimuli. At most banal, this increase is chance and meaningless. At

most interesting, these amplitude increases may result from emotional response to dreams or indicate a role of the OTR neurons in promoting transitions between states. As described previously, REM sleep can be compared to cataplexy. The increased peaks observed in transitions between states are not observed at cataplexy offset so this phenomenon is unique to REM sleep.

We measured a lot of spontaneous cataplexy during overnight recordings and much less during the social and reward tests. It is difficult to make conclusions about cataplexy in different tests because of the small number but we do see some increased activity at onset and after offset. In spontaneous cataplexy, we observe similar increased activity at onset, but increased activity an epoch before and after offset. Grouping the social cataplexy, increased activity at onset or just before is prominent. Also increased activity in the vIPAG/LPT at offset, and increased activity after offset in the CeA, but no clear peak at offset. These peaks could be attributed to generally higher activity in wake compared to cataplexy, which also means, that despite some error in determining the exact time of cataplexy transitions, the peaks are likely either at transition or just before in case of onset, or just after in case of offset. If these peaks prior to onset are not just normal wake activity, we still cannot conclude that they specifically initiate cataplexy because other similar peaks are present. In these recordings, both at low and high LED power we found lower activity in these cells during cataplexy as compared to every other state. While we do see increased activity at onset that may indicate some role in initiating cataplexy, the data does not support a role for the CeA-OTR neurons in sustaining cataplexy. What is noticeably different when social cataplexy is compared to non-social

or spontaneous cataplexy is that, despite lots of overlap and similar activity patterns, more high-amplitude activity is measured in presence of social stimulus. It is possible then there is no specific increase in activity that initiates cataplexy since we do not see cataplexy every time there is higher activity, but rather that a threshold level of activity is needed for cataplexy. So, in presence of social stimuli when it is more likely to have higher activity, it is also more likely to overcome this threshold and result in cataplexy. Thereby, high activity in social presence correlates with higher incidence of cataplexy.

However, comparing social and spontaneous cataplexy yielded very small correlation coefficients indicating very small to no correlation between these kinds of cataplexy. Which either means they are completely different, a strong and unlikely conclusion to make given the amount of noise in these traces, or that we cannot make conclusions about these patterns. The latter is more likely given also the small number of social cataplexy events recorded.

To confirm this hypothesis, future experiments should perform more Home Cage Social Tests, or longer Home Cage Social Tests, and analyze for cataplexy in conjunction with the fiber photometry to increase the number of social cataplexy episodes captured. If increased activity of these neurons correlates with higher incidence of cataplexy, this can be tested by optogenetically stimulating these neurons and their terminals which is, fortunately, already being done.

#### *Addressing Noise*

A concern, in terms of future fiber photometry recordings, is noise. Decreasing noise would eliminate ambiguity. Using a different test chamber, or shorter and more

repeated Home Cage Social Tests can help to reduce noise and allow for a cleaner signal so we only see responses from exposure to a stimulus, and can help clarify a bit better  $t = 0$ s by looking at when the barrier is lifted, when the mouse enters the chamber, and when the mouse approaches the mesh which may be a few seconds apart at most. However, it will not remove noise from the signal. We removed signals that had a clear source of noise and smoothed the signal but without a clear source of the noise we cannot remove it and be sure we have preserved the true signal.

The light intensity in these recordings was too low to sufficiently stimulate GCaMP6s so we recorded at higher excitation light power. The traces taken at higher light power output ( $0.2\mu\text{W}$ ) are more promising in terms of signal to noise ratio with traces that show clearer responses. Recordings of approach to reward items and initial social interaction, where no social interaction occurs prior to event, show responses a few seconds before contact. This also occurs on approach to social mesh post in the U-chamber social test. Increased activity is also measured near cataplexy onset, prior to transitions out of REM, and at transition from nREM to REM. Decreased activity is measured across cataplexy compared to nREM, REM, and wake. Despite the pattern of vIPAG signals appearing similar to CeA signals, with a delay of a couple seconds, correlation coefficients were extremely low indicating very high noise. Switching to higher LED power more than doubled the correlation coefficient between CeA and vIPAG traces. That confirms that the signal is stronger but there is still a substantial presence of noise. Since these were taken across overnight recordings as opposed to right around an event where we may see stronger activity, some of the patterns we see could

still be signal, although these coefficients certainly call into question all conclusions drawn. More recordings would need to be done to confirm these patterns, and those recordings should be done at higher LED power for a stronger signal. Some further adjustments to the apparatus, for example adjusting gain, or a smaller required distance between the optical fiber and target cells may help achieve a higher signal to noise ratio.

vIPAG traces tended to have lower amplitude which is expected as we are measuring fluorescence of CeA axons in the vIPAG/LPT compared to cell bodies in the CeA. The terminals are relatively thin with a smaller cytoplasm to surface area ratio as the cell bodies. Because the vIPAG signal is weaker, we do not measure magnitudes of responses quite like we can in the CeA. Nevertheless, we do see the same patterns in the CeA and vIPAG, offset about 1-3s, so we know the same patterns of activity reach the vIPAG axon terminals even though this is not the case across the long traces due to low correlation coefficients. These patterns were not as clearly present in the traces done at higher LED power despite a higher correlation coefficient which does not mean that this correlation would not be even higher were the traces to be taken simultaneously. Differences in noise that day could obscure some similarities in the pattern of traces. Future recordings would ideally be done simultaneously.

With behavioral tests, there is always concern about reproducibility. Significant changes across few trials during the U-Chamber Social Test or Home Cage Social Tests possibly would not be measured with repeat testing. The Home-Cage Social Test has a smaller number of events, and the difference in mean activity before the stimulus mouse is added in the empty chamber and social chamber during the U-Chamber test are small

and considering that these events are the same when there is no mouse present, meaningless. However, tests with high event numbers and consistent patterns with individual traces like those entering the social chamber post or approaching chocolate where we conclude that there is a response to the event with increased activity very likely would be present with continued testing, though there might be some variation in time relative to  $t = 0s$ .

Overall, these pilot studies suggest that CeA-OTR neurons respond to social and rewarding stimuli with increased activity near interaction, and the new, higher power recordings, indicate this occurs a few seconds before the interaction. CeA neurons showed increased activity prior to transitioning out of REM, and some fluorescence peaks near cataplexy onset. They are more active during wake, nREM, and REM compared to cataplexy. Most likely, the CeA neurons do not sustain cataplexy, but rather help trigger cataplexy especially in social conditions. Based on these results, CeA-OTR neurons likely participate in the link between emotion and initiation of cataplexy which should be tested further with more restrictive social testing with fiber photometry with a higher signal to noise ratio achieved at higher LED power, adjusted gain and fiber placement, or optogenetics.

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## CURRICULUM VITAE

