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Carlos R. Maestas-Olguin
University of New Mexico

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Carlos Maestas-Olgiun

Candidate

Psychology

Department

This thesis is approved, and it is acceptable in quality and form for publication:

Approved by the Thesis Committee:

Nathan S. Pentkowski , Chairperson

Jonathan Brigman

Benjamin Clark

EFFECTS OF CHEMOGENETIC INHIBITION OF VENTRAL HIPPOCAMPAL GLUTAMATERGIC NEURONS ON ANXIETY-LIKE DEFENSIVITY IN MALE LONG-EVANS HOODED RATS

By

CARLOS OLGUIN

B.S. PSYCHOLOGY
UNIVERSITY OF NEW MEXICO, 2015

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Carlos Olguin

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ABSTRACT

Previous research in rodents and humans has implicated the ventral hippocampus in regulating anxiety. However, many rodent studies examining ventral hippocampal neuronal pathways have utilized lesions that create nonspecific and/or nonreversible damage to the region. The present study sought to characterize the role of ventral hippocampal glutamatergic pyramidal neurons in modulating anxiety-like behavior during exposure to a variety of threatening stimuli. Five weeks prior to testing, male Long-Evans hooded rats received ventral hippocampal viral-vector infusions expressing either pAAV-CaMKIIα-hM4D-mCherry (DREADD) or pAAV-CaMKIIa-EGFP (GFP). DREADD transfection allowed for the specific, noninvasive and temporary inhibition of ventral hippocampal glutamatergic neurons immediately before threat presentation. Rats were evaluated for behaviors congruent with anxiety- or fear-like defensive states (e.g., freezing, risk assessment, avoidance, etc.) during testing in the elevated plus-maze and light-dark exploration test, or footshock-induced contextually conditioned fear, respectively. Analyses revealed a significant effect of DREADD inhibition that was dependent on the type of threat exposure. Specifically, compared to GFP controls, DREADD-induced silencing of ventral hippocampal glutamatergic neurons reduced anxiety-like behavior in the elevated plus-maze and light-dark test, without reliably affecting the expression of conditioned fear. The present results confirm that ventral hippocampal glutamatergic pyramidal neurons are recruited in rats during exposure to anxiety-inducing stimuli. These

data add to a growing literature implicating the ventral hippocampus as a key region involved in modulating anxiety-like behaviors in rodents, primates and humans.

Keywords: Designer receptor exclusively activated by designer drugs (DREADDs), Ca2+/calmodulin dependent kinase II, glutamatergic pyramidal neuron, defense.

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CHAPTER 1 INTRODUCTION

Normal and Abnormal Stress

The continued improvement in efficacy of psychopharmaceuticals relies, at least fractionally, on advancing our understanding of the interplay between pathological behavior and the underlying neurobiology. Some of the most debilitating psychopathologies involve altered neural systems that regulate stressor-induced behaviors during anxiety- and fear-like defensive states. Normally this biobehavioral response is healthy and adaptive allowing prey animals to avoid predation through threat detection (e.g., risk assessment) and threat avoidance (e.g., flight, freezing; Blanchard and Blanchard, 2003). This same adaptive response to stressors in humans facilitates the maintenance of allostasis and prevents the detrimental effects of a prolonged allostatic load (McEwen, 2003). However, when dysregulated, this process contributes to the manifestation of several psychopathologies commonly termed anxiety/stress disorders (de Kloet, 2003) that include generalized anxiety disorder, panic disorder, agoraphobia, specific phobias, social anxiety, obsessive compulsive disorder and post-traumatic stress disorder (American Psychiatric Association, 2013). Globally, an estimated 264 million people, or 3.6% of the population currently have an anxiety disorder (WHO, 2017) with an estimated cost of more than US$ 1.15 trillion annually (Chisolm et al., 2016). In order to improve current pharmacotherapies, future research is needed to delineate the precise roles of distinct neuronal populations in mediating stressor-induced anxiety- and fear-like defensive states.

Neurobiology of Defense

The neurobiology of defensive behavior has been explored extensively in the literature and has evolved enormously from the original “fight or flight” acute stress response construct (Cannon, 1915). The mammalian biological stress response is regulated by two intimately connected systems; the sympathetic-adrenomedullary (SAM; Frankenhaeser, 1986) system and the hypothalamic-pituitary-adrenocortical (HPA; Stratakis et al., 1995) system. Although these two systems are controlled primarily by the hypothalamus (for review, see Herman et al., 2005), regulation of stressor-induced anxiety

and fear behaviors recruit additional limbic circuits including the hippocampus, amygdala, as well as the medial and orbital prefrontal cortices (Gross & Canteras, 2012)). Initially, Klüver and Bucy (1939) implicated these regions as key structures controlling general defensive behavior when they observed that temporal lobectomies encompassing the amygdala, hippocampus and adjacent cortices produce robust behavioral alterations in rhesus monkeys. Subsequent studies reported that inhibition of the amygdala generally reduces conditioned fear-like behaviors in monkeys (Weiskrantz, 1956), rodents (Blanchard and Blanchard, 1972; LeDoux et al., 1988; Liang et al., 1992; Maren et al., 1996; Tovote et al., 2016) and humans (Terburg et al., 2012; Klumpers et al., 2015; Shackman and Fox, 2016; for general review see Phelps and LeDoux, 2005; or Tovote, Fadok and Lüthi, 2015). These findings are complimented by human functional neuroimaging studies that reveal amygdala activation during the presentation of unmasked and masked fearful visual stimuli (Morris et al., 1996; Whalen et al., 1998; Morris et al., 1999). Taken together these results indicate a critical role for the amygdala in emotional processing, particularly the expression of conditioned fear.

Septotemporal Segregation of Hippocampal Function

Historically, the pedagogy of the hippocampus has focused on spatial navigation (O'Keefe and Nadel, 1978; Morris et al., 1982), and specific forms of learning and memory (Larson and Lynch, 1986; Squire and Zola-Morgan, 1991). However, it is also undeniable that the hippocampus is involved in modulating specific anxiety-like behaviors (for review see Gray and McNaughton, 2000; Canteras et al., 2009; Strange et al., 2014). Indeed, Gray and McNaughton (2000) reported that all anxiolytic drugs modulate the behavioral inhibition system, the canon component of which is the septo-hippocampal system. These findings lead to their septo-hippocampal theory of anxiety that marked a clear distinction between fear (controlled primarily by the amygdala) and anxiety (controlled primarily by the septo-hippocampal system), and defined conflicting goals as the primary input to this system (Gray and McNaughton, 2000; McNaughton and Corr, 2004).

Lesions of the vHC

Studies performing site specific hippocampal manipulations have revealed a functional differentiation across the septotemporal axis (Risold and Swanson, 1996; Moser and Moser, 1998; Kheirbek et al, 2013), with the dorsal pole of the hippocampus (dHC) involved in various forms of memory (e.g., spatial, episodic, etc.) and the ventral pole of the hippocampus (vHC) involved in emotional regulation (for review see Fanselow and Hong-Wei, 2010; Kheirbek et al., 2013; Strange et al., 2014). For instance, Bannerman et al. (2002) found a double dissociation where NMDA lesions of the vHC but not dHC reduce anxiety-like behaviors in rats in three classic tests of anxiety [social interaction, elevated plus-maze (EPM), and hyponeophagia], while dHC but not vHC lesions produce deficits in working and spatial memory (T-maze and Morris water task). Pentkowski et al. (2006) observed that pretraining ibotenic acid vHC lesions reduce unconditioned anxiety-like behaviors in rats exposed to cat odor, but not a live cat, effects indicating a specific vHC role in regulating anxiety- but not fear-like defensive states. Importantly, matched lesions of the dHC had no effect on the expression of unconditioned anxiety-like defensive behaviors in these paradigms (Pentkowski et al., 2006). Similarly, rats with vHC, but not dHC, lesions fail to avoid the open arms of an elevated t-maze (Trivedi and Coover, 2004), and exhibit reduced measures of anxiety in the social interaction, light-dark (LDT), hyponeophagia and successive alley tests (McHugh et al., 2004). A reduction in unconditioned anxiety-like behaviors has also been observed in the EPM following vHC lesions/inactivation using lidocaine (Bertoglio et al., 2006), ibotenic acid (Kjelstrup et al., 2002), tetrodotoxin (Degroot and Treit, 2004) and electrolytic (Trivedi and Coover, 2004) procedures. Taken together, these finding clearly highlight the vHC, but not dHC, as the pivotal contributor in Gray and McNaughton's (2000) septo-hippocampal theory of anxiety.

Receptor Manipulations in the vHC

Micro-infusion studies targeting specific receptor systems further implicate the vHC in the expression of untrained defensive behaviors in response to anxiety-inducing stimuli (for review, Engin & Treit, 2007). Briefly, infusion of glutamate antagonists (Zhang et al, 2001; Hackl, Nascimento, and Carobrez, 2007; Marrocco et al., 2012), $GABA_A$ agonists (Bast et al., 2001; Trent and Menard, 2010; McEown and Treit, 2010, 2013; Zhang

et al., 2014), acetylcholine agonists (Degroot and Treit, 2002), nitric oxide (Calixto et al., 2010) and mineralocorticoid antagonists (McEown and Treit, 2011) all attenuate unconditioned anxiety-like behaviors in tasks including the EPM, shock-probe burying test, open field test, elevated T-maze, and LDT. Additionally, Pentkowski et al. (2009), reported bidirectional modulation of unconditioned anxiety-like behaviors in response to cat odor following micro-infusions of either a CRF_1 antagonist (decrease) or agonist (increase). Collectively, these micro-infusion experiments further indicate that vHC neuronal activity is required for the expression of anxiety-like defensive responses.

Genetic Manipulations of the vHC

Contemporary optogenetic and chemogenetic techniques have been developed to target specific cellular populations with anatomical and temporal specificity. Using optogenetics in mice, a recent study observed that direct inhibition of neurons projecting from the vHC to the medial prefrontal cortex (mPFC) decreases avoidance of the open arms in the EPM (Padilla-Coreano et al., 2016). Similarly, Morrone-Parfitt and colleagues utilized chemogenetics to elucidate the bidirectional control of anxiety-like behaviors by distinct vHC cell populations in the mouse. Specifically, activation of vHC neurons projecting to the lateral septum attenuated anxiety-like behaviors in EPM and a novelty suppressed-feeding task, while inhibition of this same cellular population enhanced anxiety-like behaviors (Morrone-Parfitt et al, 2017). These researchers also observed an increase in anxiety-like behaviors following selective activation of mPFC projecting vHC cells. Lastly, optogenetic activation of vHC projections to the lateral hypothalamic area increased anxiety-like behavior in mice (Jimenez et al., 2018). Collectively, these results indicate that anxiety-like behaviors in mice are controlled via the dynamic balance of signals arising from the vHC.

vHC Implicated in Conditioned Defense

In addition to modulating the expression of innate anxiety-like behaviors in response to threatening stimuli, research also implicates the vHC in modulating conditioned defensive behaviors. Following post training electrolytic lesions of the vHC, but not dHC, Trivedi and Coover (2004) observed a reduction in cue and contextual

conditioned freezing. In addition, Pentkowski et al. (2009) found bidirectional modulation of conditioned defensive behaviors during contextual re-exposure to a cat odor paired environment, where CRF_1 agonism and antagonism potentiated and attenuated defensive behaviors respectively. Similarly, pre-training ibotenic acid lesions of the vHC attenuated the expression of conditioned behaviors elicited by exposure to either cat odor-, live cat- or footshock-paired environments (Pentkowski et al., 2006). This effect was not detected during exposure to a live cat consistent with the notion that the vHC is selectively recruited during exposure to anxiety-inducing potential threats such as predator odors or conditioned contexts, rather than fear-inducing threats like a live predator (Fanselow & Lester, 1988; Blanchard & Blanchard, 1990; Kjelstrup et al., 2002). Using optogenetics, Jimenez et al. (2018) found that stimulation of vHC terminals in the basolateral amygdala impair footshock-indued contextual fear conditioning. Collectively these findings ascribe a roll for the vHC in regulating the expression of conditioned anxiety-like defensive behaviors to potentially threatening stimuli.

Purpose of study. The present study sought to replicate and extend the work by Padilla-Coreano et al. (2016), Morrone-Parfitt et al. (2017) and Jimenez et al. (2018) in the mouse by using chemogenetic techniques to investigate the functional role of glutamatergic vHC afferent neurons in regulating the expression of anxiety- and fear-like defensive behaviors in the rat. These techniques allowed us to selectively silence vHC glutamatergic neurons immediately before threat exposure. Based on the aforementioned previous work in the mouse, we predicted that silencing glutamatergic vHC neurons in the rat would attenuate anxiety-like behaviors associated with the presentation of scenarios where the potential for predation was possible, but not explicit. This work adds to the growing literature defining the function of the hippocampal complex, specifically the role of the vHC in innate and learned anxiety-like defensive behaviors.

CHAPTER 2 MATERIALS AND METHODS

Animals

Subjects were 20 male Long-Evans hooded rats bred and shipped from Charles River Laboratories (Charles River, USA). Upon arrival to the Department of Psychology Animal Research Facility at the University of New Mexico, rats were pair housed and maintained at a constant temperature (23 ℃) and diurnal cycle (12 h light/dark, lights on at 1000 h) with food and water available *ad libitum.* Rats weighed between 320 and 475 g at the time of surgery. All husbandry and procedures adhered to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and all experimental procedures were reviewed and approved by the University of New Mexico Institutional Animal Care and Use Committee.

Viral Vectors

In order to characterize the function of distinct vHC neuronal pathways, rats received viral vector infusions into the vHC to express designer receptors exclusively activated by designer drugs (DREADDs). Specifically, a pAAV-CaMKIIα-hM4D-mCherry vector system was used to selectively and temporarily silence vHC neuronal firing by inhibiting G-protein coupled receptor signaling (Dobrzanski et al 2017, Zou et al 2016 and Roth 2016). The CaMKIIα promotor allowed for the selective inhibition of vHC glutamatergic neurons, without altering cholinergic or GABAergic neuronal activity (Liu & Jones, 1996; Sík, et al., 1998), as CaMKIIα within the HC selectively colocalizes within glutamatergic neurons (Wang, et al., 2013). Additionally, pAAV-CaMKIIa-EGFP viral vectors were infused as viral controls. Viral vectors were obtained from the University of North Carolina at Chapel Hill Vector Core.

Surgery

Prior to surgery, rats were handled 4-5 times per week for three weeks to minimize stress. Rats were randomly assigned to receive either DREADD (n = 15) or GFP control (n = 5) viral infusions. Prior to surgery, rats were deeply anesthetized using 4% isoflurane and received an injection of buprenorphine (0.1mg/kg/ml, s.c.) to alleviate pain; aesthesia

was maintained throughout surgery at 1.5-2% (SomnoSuite® Low-Flow Anesthesia System, Kent Scientific). Next rats were placed into a stereotaxic apparatus (David Kopf Instruments) and received a 0.1ml injection of 2% lidocaine (s.c., Sparhawk Laboritories, 2016) under the scalp to provide local anesthesia before the surgical incision. A set of small holes (0.5 mm in diameter) were drilled bilaterally into the skull to allow for the insertion of a 10 μl micro syringe (Hamilton). Rats received two pairs of bilateral infusions within the intermediate and ventral regions of vHC using coordinates obtained from Paxinos and Watson (2012) and based on previous protocols (Pentkowski et al., 2006; 2009): ventral (-5.2 mm posterior to bregma, ±5.1 from midline and -7.1 ventral from the surface of the skull); and intermediate (-5.2 mm posterior to bregma, ±5.1 from midline and -6.0 ventral from the surface of the skull). Following each infusion (0.2μl/min; 1μl/site) the syringe remained in place for 5 additional min to allow for complete viral diffusion. Following surgery, rats received a nonsteroidal anti-inflammatory (Enfrolax® 100; Norbrook Laboritories, 2016) in their water for 3 days and were singly housed for 7 days before being reunited with their original cage mate. In order to insure complete viral expression, the virus incubated for 5 weeks before behavioral testing commenced.

Drugs

Agonists for the DREADDs [clozapine-N-oxide (CNO) and clozapine; Tocris Bioscience] were dissolved in a 0.9% sterile saline solution. Prior to testing in the EPM, rats assigned to the agonist group received injections of CNO (1mg/kg/ml, i.p.), while rats assigned to the agonist group on footshock and LDT test days received clozapine (0.1 mg/kg/ml, i.p.). CNO injections were delivered 20 minutes prior to each trial and clozapine injections were administered 30 min prior to each trial; matched saline control injections were administered using the same volume and route of injection. Thus, for each test type the following three experimental groups were obtained: DREADD/agonist (vHC inhibition, n=10), DREADD/saline (viral control; n=5) and GFP/agonist (agonist control; n=5). Dosage and injection schedules were based on previous work demonstrating that CNO can be reverse metabolized to clozapine and produce clozapine-like interoceptive stimulation at doses larger than 1mg/kg after 30 min (Gomez et al., 2017; Manvich et al., 2018). Both agonist compounds were utilized to determine if the same behavioral effect

could be achieved with both compounds using different tests of anxiety to rule out possible reverse metabolism as a confound.

Behavioral Testing

All behavioral testing occurred between 1000 and 1400 h in the following order: EPM, footshock-induced contextual fear conditioning and LDT; each test was separated by 7-12 days to prevent potential additive effects of stress and to prevent agonist accumulation between tests. Anxiety- and fear-like defensive behaviors were analyzed during exposure to a potentially threatening environment (EPM and LDT) as well as immediately following threat exposure (post footshock). Additionally, a contextual conditioned test was performed 24 h after initial footshock exposure. Each apparatus was cleaned using a 10% ethanol solution between trials.

Behavioral Measures

Anxiety- and fear-like defensive behaviors are species typical and situation specific (Bolles, 1970; Blanchard and Blanchard, 1989). Although the defensive states of anxiety and fear are related, they are discrete categories, elicited by specific conditions, and represented by unique sets of behaviors (Perusini & Fanselow, 2015). Indeed, anxiety- and fear-like defensive states are so closely related that the terms are often mistakenly used interchangeably, identical behavioral indices are used to measure them, or one term is used to define the other. Even the Diagnostic and Statistical Manual of Mental Disorders, 5th ed. (DSM-V; American Psychology Association, 2013) lists the essential characteristic of agoraphobia, an anxiety disorder, as the "intense fear of any place or situation where escape might be difficult." This lack of consistency throughout the literature hinders the translation between clinical practice and pre-clinical research. In order to avoid this ambiguity, the present study adopted the Predator Imminence Model (Fanselow & Lester, 1988). This model defines the antecedent causes (pre-encounter, post-encounter and circa-strike) and elicited behaviors (risk assessment, freezing, biting, etc.) that differentiate anxiety from fear, and in a more recent review was demonstrated to align with the National Institute of Mental Health's adoption of the Research Domain Criteria (for review see, Perusini & Fanselow, 2015). Thus, the EPM and LDT were utilized to simulate pre-encounter

situations that elicit anxiety-like behavior characterized by changes in exploratory and risk assessment behaviors. In contrast footshock conditioning and contextual re-exposure were used as an analogue for post-encounter situations that elicit fear, characterized predominantly by freezing in rodents (Blanchard and Blanchard, 1969). The post footshock and contextual fear conditioning trials measured levels of *freezing*—complete cessation of movement other than respiration. During the EPM and LDT trials, measures included: *head outs*—extension of the head out of the closed arm (EPM) or dark compartment (LDT) without exciting the location; *transits*—crossing from one chamber into the other chamber of the apparatus (LDT), or the number of open and closed arm entries (EPM), measured as all four paws moving from one marked section of the apparatus to another; and *avoidance*—duration of time spent in the dark compartment (LDT), or the percentage of time spent in the closed versus open arms (EPM). Additional behavioral measures scored in the EPM test included: *head dips*—extension of the subject's head over the edge of an open arm; and *risk assessment*—combined measure of both *stretch approach*—forward ambulation with flat back and stretched neck and *stretch attend*—standing on all four paws with flat back and stretched neck orientated toward the threat source. Behavioral measures represent the durations of events in seconds, numbers of transits, or percentages of total time in a 5 min observation period for each test.

Elevated Plus-maze

All sessions were conducted under red light. The EPM apparatus consisted of four Plexiglas arms arranged to form a plus, elevated 75 cm above the floor. Each arm was 10 cm wide and 50 cm long, and each arm was joined at the center by a 10 cm square platform. The two opposite "open" arms contained no walls, while the other two "closed" arms had 40 cm high side walls. The front panel of each enclosed arm was made of clear Plexiglas to allow for observation and recording. Twenty min prior to testing, control [n=10; GFP/CNO (n=5) and DREADD/saline (n=5)] and experimental (n=10; DREADD/CNO) rats received their assigned injections and were placed in the center of the apparatus facing one of the closed arms.

Footshock

All sessions were conducted under dim, white light. The footshock apparatus consisted of a Plexiglas/metal enclosed chamber (Coulbourn Instruments; 30 x 25 x 30 cm) with a clear front panel to permit recording. Test chambers were equipped with a metal bar floor that was connected to a manual precision animal shocker (Coulbourn Instruments). On unconditioned test days all rats received their assigned injections 30 min prior to the start of their test trial: control [n=10; GFP/clozapine (n=5) and DREADD/saline (n=5)] and experimental (n=10; DREADD/clozapine) rats. Once the trial commenced, rats were allowed to explore the apparatus for 3 min before receiving three 1mA scrambled footshocks administered 1 min apart; fear-like defensive behaviors were measured for 5 min following the administration of the final shock (post footshock test). Rats were returned to the same test chamber 24h after the unconditioned test trial for a contextual conditioned test trial. No injections were administered on the conditioned test day.

Light/dark Test

All sessions were conducted under dim, white light. The LDT apparatus consisted of two adjacent Plexiglas compartments (30 x 30 x 60 cm), one was white with no top and one was black with a black lid to prevent light entry. The light and dark compartments were separated by a Plexiglas wall that had white on one side and black on the other, with an open partition that allowed for the animal to freely move from one side to the other. The apparatus was positioned so that the white compartment was centered below an overhead light source. Thirty min prior to testing, control [n=10; GFP/clozapine (n=5) and DREADD/saline (n=5)] and experimental (n=10; DREADD/clozapine) rats received their assigned injections and were placed in the dark compartment of the apparatus.

Histology

Following completion of behavioral testing, histological verification of viral expression was performed. Rats were deeply anesthetized with Fatal Plus (1ml/kg, i.p.) and were perfused transcardially with a 0.1M ice cold phosphate buffered saline followed by fresh ice cold 4% paraformaldehyde. Following extraction, brains were placed in 4% PFA for 24h before being transferred to a 15% sucrose solution for cryoprotection. The brains remained in this solution until they visibly sank (approximately 24h) and then were

transferred to a 30% sucrose solution where they remained (at least 24h) until sectioning on a cryostat (Thermo Scientific). A series of coronal sections (40 μm) were collected throughout the entire anterior-posterior range of the dHC and vHC and were stored in a cryoprotectant solution until immunostaining.

Immunohistochemistry

In order to confirm localized vHC viral expression, sections from each DREADD-infused rat were immunostained for mCherry; GFP was visualized without immunostaning. Free-floating sections were first washed in 0.1M PB to remove the cryoprotectant followed by 50mM ammonium chloride. Next, the tissue was soaked in PB containing 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) and 0.3% Triton X-100 followed by a 48h incubation in the mouse monoclonal anti-mCherry serum (1:500, Abcam, ab125096) containing 0.1% bovine serum albumin (Sigma, a9647) 3% NGS, and 0.3% Triton X-100. Subsequently, the tissue was washed in PB and incubated for 2h at room temperature with the biotinylated goat anti-mouse AlexaFlour-594-labeled antibody (1:500, Thermo Fisher Scientific, A21145). Next, sections were washed in PB and cover-slipped with mounting media containing DAPI (Vector, H1200. Slides were analyzed on a Leica DMRXA2 epifluorescent microscope (Leica) equipped with a mercury lamp for mCheery and GFP fluorescent imaging. To assess placement and spread of the viral infusions, sections were compared to a schematic representation from the Paxinos and Watson brain atlas (2012). Rats with misplaced viral expression or that lacked viral expression entirely were excluded from the analyses.

Statistics

Independent sample Student's t-tests and Levine's tests for equality of variance were performed on each unconditioned and conditioned dependent measure. Alpha was set at 0.05 for all comparisons. We predicted there would be no observed differences between the GFP/CNO/clozapine and the DREADD/saline rats in any behavioral test and therefore data from these two groups were compared first, with the intention of pooling their data into one control group. Thus, final statistical comparisons were planned between controls

(GFP/CNO/clozapine + DREADD/saline) and experimental (DREADD/CNO/clozapine) rats for each dependent variable.

CHAPTER 3 RESULTS

Histology

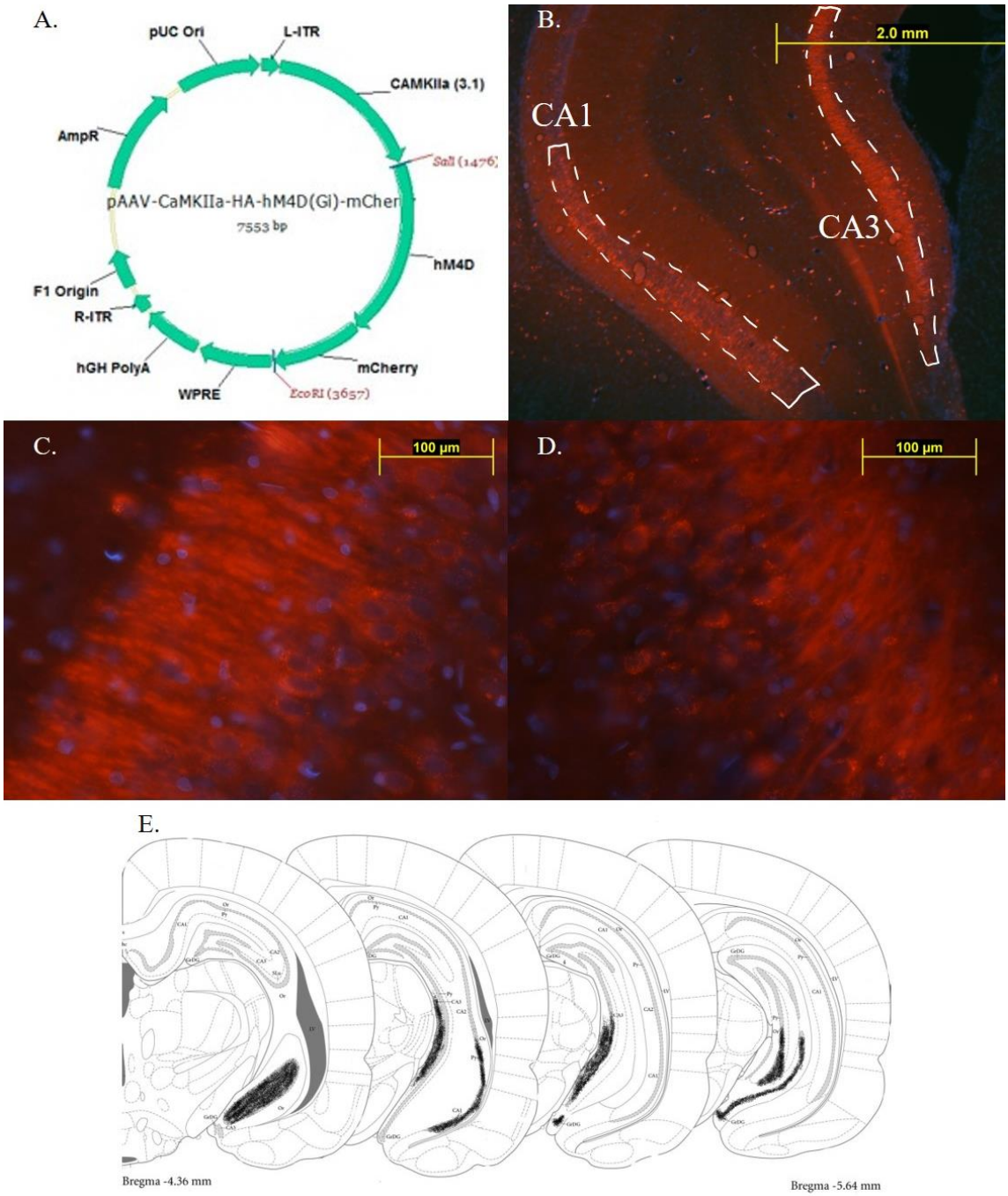

Fig. 1. CAMKIIα mediated DREADD expression delivered via viral vector (A), produced robust receptor expression in the vHC (4x magnification; B). Photomicrographs of representative expression within the ventral CA1 and CA3 are shown (40x magnification; C and D, respectively). Serial histological reconstruction depicting the spread of CAMKII mediated DREADD expression throughout the vHC (E).

A serial histological reconstruction of the viral-mediated gene expression within the vHC is shown in **Fig. 1.** Visual examination of each section revealed that no rat

exhibited viral expression outside of the vHC, with expression ranging between -4.36mm and -5.64mm posterior to bregma. All rats exhibited expression throughout the CA3 field of the vHC (-4.36mm and -5.64mm posterior to bregma), and within the CA2 and CA1 fields of the vHC (-4.56mm and -4.80mm posterior to bregma). Fluorescently tagged fiber tracts were observed throughout the oriens layer of the vHC (-4.44mm and -5.52mm posterior to bregma), and positively marked cells were observed within the pyramidal cell layer of the vHC (-4.80mm and -5.16mm posterior to bregma) and the granular layer of the dentate gyrus of the vHC (-5.40mm and -5.64mm posterior to bregma).

Elevated Plus-maze

The durations and frequencies of each dependent measure during testing in the EPM for both the experimental and control groups are presented in **Fig. 2.** As predicted, comparison between DREADD/saline viral-controls and GFP / CNO / clozapine agonist-controls revealed no statistically

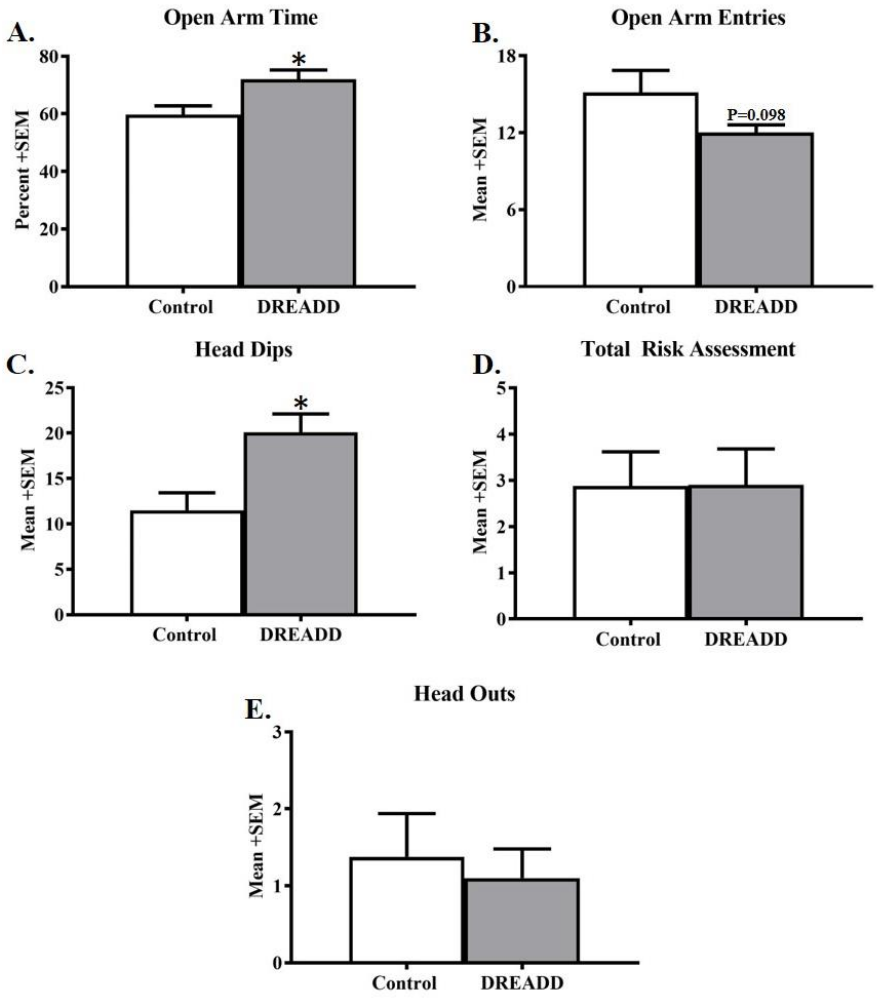

Fig. 2. Effects of chemogenetic vHC inhibition (mean + SEM) on anxiety-like behaviors in the EPM. Exploratory (A, B, and C) and risk assessment (D and E). Animals received their assigned dose of saline or CNO 20 min prior to testing. Differences for which *p < 0.05.

significant differences between their respective scores on any of the behaviors measured (data not shown). Thus, these groups were pooled into one control group and all subsequent analysis was performed between the pooled control group (DREADD/saline + GFP/CNO/clozapine) and the experimental (DREAD/CNO/clozapine) group. One control rat was removed from this analysis because at least two of its measures deviated by greater than three standard deviations from the mean. Thus, final statistical analysis for the EPM was conducted on the nineteen remaining rats. There was a significant increase in both the percent of open arm time (M=0.7209, SD=0.101) and the number of head dips (M=20.10, SD=6.35) following vHC chemogenetic inhibition compared to controls (M=0.5737, SD=0.1066 and M=10.56, SD=5.855); $t(17)=-3.088$, $p < 0.05$ and $t(17)=-3.393$, $p < 0.05$, respectively. There was a trend towards a decrease in open arm entries for the inhibition group; $t(17)= 1.852$, $p=0.098$. No significant group differences were detected for head outs or total risk assessment measures ($p > 0.05$ in each case).

Footshock

Freezing durations for both the vHC-inhibition and control rats for the post-shock and context re-exposure test trials are presented in **Fig. 3.** One rat was withheld from this behavioral test due to an injury that inhibited

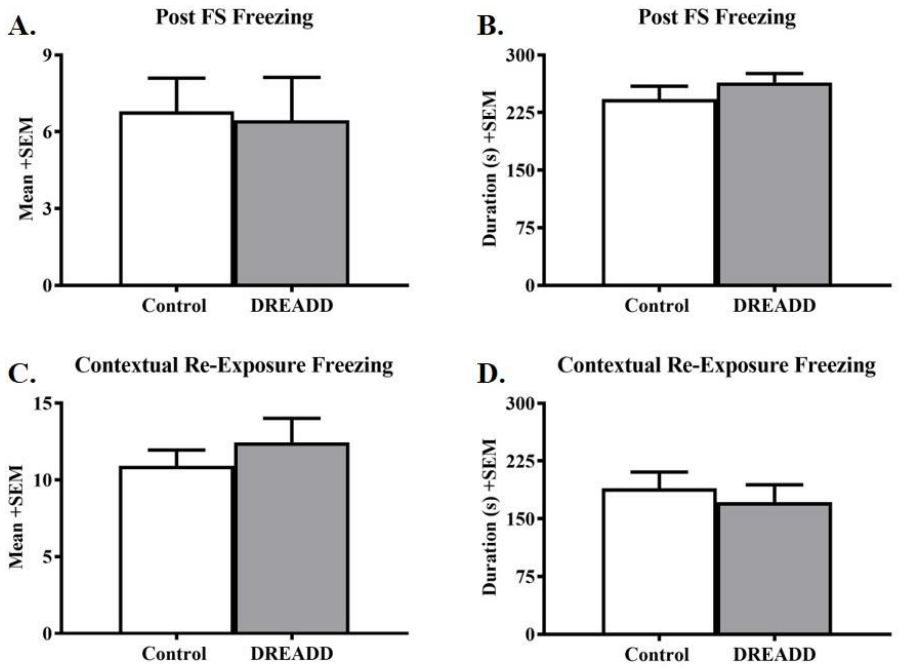

Fig. 3. Effects of chemogenetic vHC inhibition (mean + SEM) on fear-like behavior in the footshock (A and C) and contextual re-exposure tasks (B and D). Animals received their assigned dose of saline or CLZ 30 min prior to testing. No statistically significant differences observed.

locomotion. No significant differences in freezing were detected between groups ($p > 0.05$).

Light/dark Test

Fig. 4 presents the durations and frequencies of each dependent measure during the LDT. Two experimental rats were removed from the analysis; one rat escaped the apparatus during testing and the other rat was removed because it developed an injury prior to test day that inhibited locomotion. Chemogenetic inhibition of the vHC significantly increased the number of light entries (M=3.88, SD=1.727) compared to controls (M=1.60, SD=1.506); $t(16)=-2.986$, $p = 0.05$. There was also a significant increase in the duration of time spent in the light chamber following vHC inhibition (M=93.80, SD=27.735) compared with controls (M=46.28, SD=50.311); $t(16)=-2.543$, $p < 0.05$. No significant group differences were detected in latency to enter the light chamber or the number of head outs ($p > 0.05$ in each case).

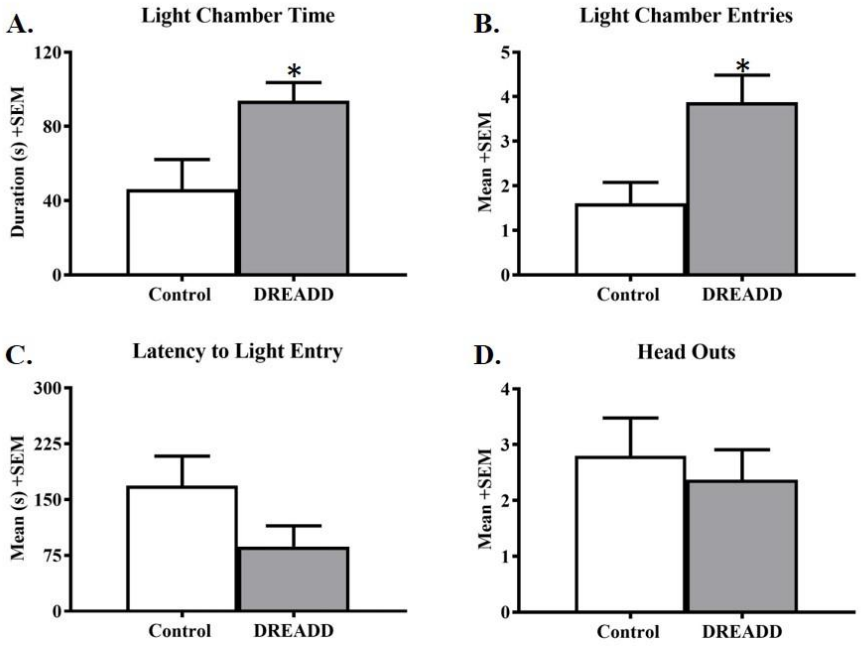

Fig. 4. Effects of chemogenetic vHC inhibition (mean + SEM) on anxiety-like behaviors in the LDT. Animals received their assigned dose of saline or CLZ 30 min prior to testing. Differences for which *$p < 0.05$.

CHAPTER 4 DISCUSSION

vHC Glutamatergic Output Required for Anxiety-Like Behavior in Rats

The present results are the first to describe a unique role for vHC glutamatergic output neurons in rats as mediators of defensive responses arising from pre-encounter instances that provoke anxiety-like behaviors, but not post-encounter instances that provoke fear-like behaviors. This work replicates and extends previous work in mice that found similar effects on anxiety- but not fear-like behaviors following inhibition of vHC glutamatergic pathways (Padilla-Coreano et al., 2016; Morrone-Parfitt et al., 2017; Jimenez et al., 2018), as well as pharmacological antagonism of vHC glutamate receptors in mice (Zhang et al, 2001) and rats (Hackl, Nascimento, and Carobrez, 2007; Marrocco et al., 2012; Zhu et al., 2018). Specifically, we observed an increase in exploratory behavior of novel, potentially threatening environments (EPM and LDT) following chemogenetic inhibition of vHC glutamate neurons, while post-shock and contextual fear-like behavior remained unaltered. These observations are consistent with previous literature defining anxiety and fear as discrete phenomena necessarily controlled by discrete neural pathways (Blanchard and Blanchard, 1989; Perusini & Fanselow, 2015). These results confirm that vHC glutamatergic pyramidal neurons are part of the neural circuitry that control defensive behaviors evoked by anxiogenic stimuli in rats.

The vHC consists of several heterogeneously distributed cell types including glutamatergic, GABAergic, and cholinergic neurons (Siegel et al., 1994; Freund & Buzsaki, 1996; Nomura et al, 1997; Spencer & Bland, 2019), and although CAMKIIα has been shown to selectivity colocalize with glutamatergic neurons (Wang et al., 2013), there

remains the possibility that this association is not exclusive within the vHC. However, transgenic and pharmacological inhibition of GABAergic hippocampal interneurons have resulted in a schizophrenia-like behavioral profile (for review, see Heckers & Konradi, 2015). Likewise, neuroimaging and post-mortem analysis of schizophrenic patients revealed hippocampal hyperactivity and stark volume reduction due to atrophy of GABAergic interneurons (Konradi et al, 2001; Benes, F.M., 2015). Furthermore, anxiety-like behaviors and the spatial representation of open arm aversion in the EPM, along with vHC-mPFC theta synchrony are dependent on excitatory, but not inhibitory input from the vHC (Padilla-Coreano et al., 2016). Similarly, cholinergic cells within the hippocampus have been repeatedly implicated in facilitating learning and memory through the processes of long-term potentiation and long-term depression (Ovsepian et al., 2004; for review, see Palacios-Filardo &Mellor, 2019). We observed no deficits in learning/memory and no symptoms of psychosis, and therefore we are confident that the results obtained were the consequence of a glutamatergic-specific manipulation.

Limitations of the Present Study

Several methodological limitations in the present study should be noted. First, DREADD-induced vHC inhibition only occurred prior to the footshock conditioning trial. While our data in rats and previous reports in mice (Morrone-Parfitt et al., 2017) suggest that vHC glutamatergic neurons are not necessary for acquiring post-encounter and contextual post-encounter fear-like behaviors, we cannot rule out the possibility that these neurons may be involved in consolidation or recollection processes. Second, our contextual conditioning environment may not have been complex enough to require a hippocampal

representation of the environment (Wang et al., 2013), and thus conditioned freezing may have been driven by the amygdala (Tovote et al., 2016).

Future Directions

In order to further delineate the precise role of the vHC in regulating defensive behaviors in rats, future studies should examine specific vHC efferent pathways, as well as additional vHC cell populations. Specifically, studies should explore whether vHC glutamatergic neurons in rats bi-directionally regulate anxiety-like behaviors via separate septal and cortical (Morrone-Parfitt et al., 2017) or hypothalamic (Jimenez et al., 2018) pathways, which have been reported in mice. Similarly, future research is needed to examine the role of specific connections between the vHC, mPFC, and amygdala (Ishikawa & Nakamura, 2006; Kim & Cho, 2017) on anxiety- and fear-like defensive behaviors. Lastly, the effects of chemogenetic and/or optogenetic manipulations of additional vHC cell types (e.g., GABAergic, cholinergic, etc.) on anxiety- and fear-like behaviors are needed to fully characterize the role of the vHC in mediating defense.

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