DISSERTATION

SEXUAL DIVERGENCE IN PREFRONTAL NEURAL REGULATION AND ENCODING OF DEPRESSION-ASSOCIATED BEHAVIORS

Submitted by

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In partial fulfillment of the requirements

For the Degree Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2022

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ABSTRACT

SEXUAL DIVERGENCE IN PREFRONTAL NEURAL REGULATION AND ENCODING OF DEPRESSION-ASSOCIATED BEHAVIORS

Major depressive disorder (MDD) accounts for the most years lived with disability worldwide. Yet, despite its staggering prevalence, the biological mechanisms underpinning MDD onset are not understood, further complicated by considerable sex-based differences in MDD occurrence. The ventromedial prefrontal cortex (vmPFC) is heavily associated with MDD, though how vmPFC neural populations respond to and regulate behaviors associated with MDD, including affective state, social behaviors, and stress responding is unknown. Thus, I utilized viral methods to dissect how a genetically identified neural population within the vmPFC regulates and encodes MDD-associated behaviors. In chapter 2, I utilized an optogenetic technique to increase the firing rate of a subset of glutamatergic vmPFC neurons in conjunction with behavioral testing. My results demonstrated considerable sexual divergence in vmPFC glutamatergic influence. In males, stimulation conferred positive affect, increased social motivation, and constrained aspects of the acute stress response. While in females, stimulation did not alter behavior and augmented the acute stress response. In chapter 3, I utilized a similar optogenetic technique to dissect how vmPFC projections to the posterior hypothalamus (PH), contribute to behavioral and physiological regulation. Again, my results demonstrated sexual divergence in vmPFC circuit function. In males, stimulation of the vmPFC to PH glutamatergic circuit conferred positive affect, and constrained aspects of the acute stress response, though it

did not alter social behavior. The circuit similarly conferred positive affect in females, but again augmented the acute stress response. Overall, my stimulation of vmPFC glutamatergic neurons identified that they regulate affect, social behavior, and stress responding but the specific effects are sex and circuit specific. While chapters 2 and 3 identified how specific vmPFC neural populations can regulate behavioral and physiological processes, how these neural populations respond to behavior and how these responses are disrupted in pathology was unknown. Thus, in chapter 4, I utilized fluorescent calcium indicators to record the activity of genetically-identified vmPFC glutamatergic neurons during behavioral testing. To determine changes to vmPFC neural activity in pathology, animals were exposed to a preclinical model of MDD, chronic variable stress. My results showed that vmPFC glutamatergic neurons are responsive to object, social, stressful, and rewarding stimuli regardless of sex. However chronic stress exposure altered vmPFC glutamatergic activity in males more so than females, with some of these differences accounted for by female ovarian status. Overall, the work presented in this dissertation determined how a vmPFC neural population regulates MDD-disrupted behaviors, detailed how a specific vmPFC circuit contributes to this regulatory role and measured how vmPFC neurons respond to behavior in real-time with and without a history of chronic stress.

ACKNOWLEDGEMENTS

Brent: The road to finishing these studies has been filled with countless difficulties for both of us. Yet, throughout the problems you have maintained a steadfast dedication to your principles and a fervor for the advancement of science. This dedication will always impress and inspire me. Thank you for all your help and guidance.

Drs. Hentges, Amberg, and Conner: Thank you all for offering your support and for keeping me grounded and moving forward. Without your help I would have spent more time missing the forest for the trees.

Sebastian: Thank you for the jokes, the puns, and the generally calm demeanor that helped the lab keep putting one foot in front of the other. I have watched you become an excellent scientist and an even better father.

Derek: Sharing major projects is not always an easy task. Yet, I am proud of how well we came together to accomplish large and complex studies. I could always trust that even if the path got murky you would always be there to help carry the work. Whatever you chose to do I know you will excel at.

Mom: Starting a Ph.D. after losing dad made the path even more difficult, but I always had your support. You may not have always known what I was doing, but you always let me know you cared and that made a world of difference.

Dad: Though you may be gone, thank you for teaching me the value of hard work and a good laugh. You showed me the importance of being genuine and honest. I miss you greatly.

Lainey: We made it through high school, being first-generation college students, and now graduate school together. Your support has been my joy. I've watched you become a deeply compassionate veterinarian and I could not be prouder of you. While I might not know what is next, I know that we will face it together and that is enough. There's also sure to be far too many dogs and cats about.

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CHAPTER 1: INTRODUCTION

Preamble

The following material was published online August 26th, 2021 in Frontiers in Behavioral Neuroscience under the title "Effects of Biological Sex and Stress Exposure on Ventromedial Prefrontal Regulation of Mood-Related Behaviors" (Wallace and Myers, 2021).

Summary

The ventral portion of the medial prefrontal cortex (vmPFC) regulates mood, sociability, and context-dependent behaviors. Consequently, altered vmPFC activity has been implicated in the biological basis of emotional disorders. Recent methodological advances have greatly enhanced the ability to investigate how specific prefrontal cell populations regulate mood-related behaviors, as well as the impact of long-term stress on vmPFC function. However, emerging preclinical data identify prominent sexual divergence in vmPFC behavioral regulation and stress responsivity. Notably, the rodent infralimbic cortex (IL), a vmPFC subregion critical for anti-depressant action, shows marked functional divergence between males and females. Accordingly, this review examines IL encoding and modulation of mood-related behaviors, including coping style, reward, and sociability, with a focus on sex-based outcomes. I also review how these processes are impacted by prolonged stress exposure. Collectively, the data suggest that chronic stress has sex-specific effects on IL excitatory/inhibitory balance that may account for sex differences in the prevalence and course of mood disorders.

Background

Negative mood states are a feature of numerous psychiatric conditions, including anxiety and depressive disorders. Furthermore, major depression, characterized by sadness, reduced motivation, and anhedonia, is the leading cause of years lived with disability worldwide (Friedrich, 2017). Although females are disproportionally impacted by mood disorders, preclinical studies have historically focused on male neural regulation of depression-related behaviors (Kuehner, 2017). However, recent policy and methodological advances have led to the discovery of significant sex differences in the neurobiology of mood. Here, we examine recent studies exploring sex differences in the prefrontal regulation of coping, reward, and sociability.

Clinical neuroimaging studies associate activity in the ventral medial prefrontal cortex (vmPFC) with depressive disorders, and emotional regulation broadly. The vmPFC is also essential for goal-directed and contextually-appropriate behaviors, mood, and stress responding (Nestler et al., 2002; Krishnan and Nestler, 2008; McKlveen et al., 2015). Further, in studies of males and females vmPFC activity is linked with reward processing and positively correlates with the severity of anhedonia (Keedwell et al., 2005; Green et al., 2019). In particular, the vmPFC subregion Brodmann Area 25 (BA25) has decreased volume in MDD patients across sexes, and is a target for deep brain stimulation in treatment-resistant depression (Drevets et al., 1997, 2008; Crowell et al., 2019; Sankar et al., 2019). A meta-analysis of imaging studies using males and females revealed BA25 is responsive to reward and emotional processing (Beckmann et al., 2009), as well as social exclusion (Vijayakumar et al., 2017). Though these studies indicate BA25 activity associates with depressive disorders, results examining BA25 function in depression are varied. Mixed sex studies have reported both reduced metabolic activity in MDD patients (Drevets et al., 1997), as well as hyperactivity in treatment-resistant depression

measured by cerebral blood flow (Mayberg et al., 2005). Yet, both conventional antidepressant treatment and deep brain stimulation reduce BA25 activity (Mayberg et al., 2005, 2013). The heterogeneity of neural populations in BA25 may contribute to these divergent results. BA25 is principally composed of excitatory pyramidal neurons that project throughout limbic and brainstem nuclei, with a smaller but diverse population of inhibitory interneurons (Beckmann et al., 2009). Mounting evidence indicates that changes to the excitation/inhibition balance of the vmPFC relate to depressive symptomology, but the contributions of specific neural populations to behavior are difficult to address clinically (Fogaça and Duman, 2019; McKlveen et al., 2019). Further, although clinical studies commonly include both males and females, few analyze outcomes for sex differences (Vijayakumar et al., 2017). However, recent advances in neurobiology have allowed studies to establish casual roles for specific genetically defined cell populations for processing and regulating behavior across sexes.

The infralimbic cortex (IL) is the rodent anatomical homolog of primate BA25 and is well-positioned for behavioral regulation based on projections throughout the limbic system. (Vertes, 2004; Wood et al., 2018). The IL contains glutamatergic projection neurons with inhibitory interneurons providing local network regulation (McKlveen et al., 2015, 2016; Wood et al., 2018). While pharmacological and lesion studies have linked IL activity with depression-relevant behaviors, advances in cell-type specificity have identified sex-dependent roles in stress, reward, and social processes.

Negative valence: stress coping

Coping with negatively-valenced stimuli involves coordinated behavioral and physiological responses to address real or perceived stressors. Ultimately, stress exposure

initiates a neurohormonal cascade that leads to the synthesis of adrenal glucocorticoids that then provide feedback to the brain at glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) to promote behavioral and physiological adaptation (McKlveen et al., 2013; Myers et al., 2014; Herman et al., 2016). An increasing number of studies have identified a role for the IL in stress coping (Table 1), with similarities and differences between male and female rodents.

The IL is acutely stress-responsive, as identified by histological markers of neuronal activation, and expresses both GR and MR in multiple cell types (Granholm et al., 1985; Reul and De Kloet, 1986; Cintra et al., 1994; Cullinan et al., 1995; McKlveen et al., 2013, 2016, 2019). Knockdown of male IL GR expression increases passive coping in the forced swim test (FST) and glucocorticoid responses to acute stress (McKlveen et al., 2013). However, pharmacological manipulations of male IL activity have yielded mixed results on coping behaviors. For instance, IL inactivation via $GABA_A$ receptor activation reduces FST immobility in males, an antidepressant-associated phenotype (Slattery et al., 2011). Similarly, non-specific synaptic blockade in the male IL reduces passive coping in the FST (Scopinho et al., 2010), as does long-term knockdown of IL glutamatergic output in males (Pace et al., 2020). However, both the NMDA antagonist ketamine and the muscarinic antagonist scopolamine increase IL activity and reduce male FST immobility (Fuchikami et al., 2015; Navarria et al., 2015). Further, deep brain stimulation in male rodents reduces passive coping in the FST (Hamani et al., 2010) and increases open arm time in the elevated plus maze (EPM), an anxiolytic-like phenotype (Shimizu et al., 2018). The mixed outcome of pharmacological interventions highlights the need to determine endogenous neural activity patterns during behavior, as well as more temporally- and genetically specific modulation of neural activity.

In vivo recordings indicate the male IL processes anxiogenic stimuli and acute stressors. IL neural activity, measured by multiunit electrodes, increases in the seconds preceding entry into the open arms of the EPM (Shimizu et al., 2018). More generally, multiunit electrode array recordings of male mPFC neurons at the border between the IL and prelimbic (PL) area have variable activity in response to FST. Although many neurons are inhibited during FST, a large portion have selectivity for immobile vs. mobile periods and the majority of those increase activity during mobile periods (Warden et al., 2012). The heterogeneity of cellular responses underscores the need to examine the contributions of specific IL neural populations. Advances in optogenetics have permitted temporally precise and cell-type specific modulation of IL activity. Activation of male IL glutamatergic neurons twenty-four hours before testing reduces FST passive coping (Fuchikami et al., 2015), suggesting IL stimulation induces pyramidal neuron plasticity. Although, it remains to be determined how this stimulation may regulate IL efferent activity. Optogenetic modulation also permits synaptic stimulation, which indicates output targets differentially influence behavioral outcomes. For instance, Warden et al. stimulated male mPFC (IL and PL) glutamatergic neurons without affecting FST behavior. However, evoked glutamate release from mPFC terminals in the dorsal raphe increased active coping, while projections to the lateral habenula decreased active coping (Warden et al., 2012). Taken together, these results indicate differing behavioral outcomes from modulating male IL activity, likely relating to the cellular specificity of interventions and/or the differential engagement of output targets.

In line with regulating coping behavior, the IL also mediates physiological responses to acute challenges. Viral-mediated knockdown of male pyramidal neuron vesicular glutamate transporter 1 (vGluT1) reduces glutamate release and increases hypothalamic-pituitary-adrenal

(HPA) axis, heart rate, and blood pressure responses to restraint (Myers et al., 2017; Schaeuble et al., 2019). Similarly, optogenetic stimulation of male IL glutamatergic neurons reduces both corticosterone and glucose responses to restraint stress, as well as heart rate and blood pressure responses to a novel environment. In contrast, optogenetic stimulation of female IL glutamate neurons increases glucose responses to restraint and heart rate reactivity to a novel environment (Wallace et al., 2021). Collectively, these data suggest that male IL glutamatergic neurons are both necessary and sufficient to reduce autonomic and neuroendocrine responses to stress, while female IL glutamate neurons facilitate stress reactivity. It remains to be determined what mechanisms account for sex differences in IL function. While IL c-Fos expression following FST is similar in males and females, males have greater activation following acute restraint, suggesting differences in stress reactivity may be stimuli-specific (Sood et al., 2018). Further, ovarian hormones may be involved as lateral ventricle infusion of corticotrophin releasing hormone leads to negative correlations between IL c-Fos expression and grooming behavior in both male and diestrus female rats. However, IL activity positively associates with grooming in proestrus females (Wiersielis et al., 2016). Overall IL neural populations signal distinct aspects of stressors, while male IL glutamatergic neural activity constrains the physiological stress response and bidirectionally regulates coping style depending upon projection sites. In contrast, female IL glutamatergic neural activity facilitates the physiological stress response and divergent IL responses to stressors may relate to ovarian hormone signaling.

Table 1: Negatively valenced stimuli. IL effects on coping during acute stressors.

Reference	Sex	Species	IL Manipulation	Timing	Stressor	Outcome
McKlveen et al. 2013	Male	Sprague-Dawley	shRNA GR knockdown	5-6 weeks before testing	FST and restraint stress	FST immobility ↑ RS corticosterone ↑
Slattery et al. 2010	Male	Sprague-Dawley	Muscimol	10 min before testing	FST	Immobility ↓
Scopinho et al. 2010	Male	Wistar	$CoCl_2$ synaptic blockade	10 min before testing	FST	Immobility ↓
Pace et al. 2020	Male	Sprague-Dawley	siRNA vGluT1 knockdown	6 weeks before testing	FST	Immobility ↓
Fuchikami et al. 2015	Male	Sprague-Dawley	Ketamine and muscimol CaMKII-ChR2 stimulation: 15 ms, 10 Hz, 5 mW	24 hours before testing	FST	Ketamine: immobility ↓ Ketamine + muscimol: no change ChR2 stim: immobility ↓
Navarria et al. 2015	Male	Sprague-Dawley	Scopolamine and muscimol	24 hours before testing	FST	Scopolamine: immobility ↓ Scopolamine + muscimol: no change
Hamani et al. 2010	Male	Sprague-Dawley	Electrical stimulation	After first FST. prior to second	FST	Immobility ↓
Shimizu et al. 2018	Male	Sprague-Dawley	Electrical stimulation	During behavior	EPM	Open arm time ↑
Warden et al. 2012	Male	Long-Evans rats	CaMKII-ChR2 stimulation: 5 ms, 20 Hz, 10-20 mW	During behavior	FST	mPFC stim: no change mPFC to DRN: immobility ↓ mPFC to habenula: immobility ↑
Myers et al. 2017	Male	Sprague-Dawley	siRNA vGluT1 knockdown	6 weeks before	RS	ACTH ↑ Corticosterone ↑
Schaeuble et al. 2019	Male	Sprague-Dawley	siRNA vGluT1 knockdown	6 weeks before	RS	Heart rate ↑ Blood pressure ↑
Wallace et al. 2021	Male and Female	Sprague-Dawley	CaMKII-ChR2 stimulation: 5 ms, 10 Hz, 3 mW	During behavior	RS and novel environment (NE)	**Males:** RS: corticosterone and glucose ↓ NE: heart rate and blood pressure ↓ **Females:** RS: glucose ↑ NE: heart rate ↑

Positive valence: reward

The IL has a prominent role in coordinating context-appropriate reward-seeking behaviors (Table 2). Pharmacological inhibition of the male rat IL with combined $GABA_A$ and

$GABA_B$ agonists reduces inhibitory control in a food reward-seeking task (Capuzzo and Floresco, 2020), as well as extinction and renewal of context-conditioned food reward (Eddy et al., 2016). Moorman and Aston-Jones (2015) used a similar pharmacological approach and found that IL inhibition reduces both lever presses to a reward-associated stimulus and extinction of reward-seeking after the stimulus is no longer paired with reward. Furthermore, male IL multiunit potential recordings found that putative pyramidal neurons heterogeneously respond (significantly increase or decrease activity) to cue-evoked reward-seeking and extinction (Moorman and Aston-Jones, 2015). Additionally, male IL neurons have prolonged firing in response to rewarded but not unrewarded operant responses and IL inhibition increases the latency to reward acquisition (Burgos-Robles et al., 2013). IL pyramidal neuron regulation of midbrain dopamine signaling may be important for effects on reward and motivation. Ferenczi et al. utilized a stable-step function opsin (SSFO) to optogentically increase male IL glutamate neuron excitability and found reduced sucrose preference. In females, the SSFO approach also reduces the rewarding quality of ventral tegmental area stimulation in a real-time place preference assay (Ferenczi et al., 2016), suggesting IL inhibition of dopamine signaling may contribute to anhedonia. In contrast, SSFO-induced increases in the excitability of IL GABAergic/vasoactive intestinal peptide (VIP)-expressing interneurons reduces high-calorie palatable food consumption without impacting food reward motivation or low-calorie chow intake (Newmyer et al., 2019). Overall, these studies indicate that male IL neurons signal multiple aspects of food reward acquisition, including contextually appropriate reward-seeking and behavioral inhibition. Although, specific IL cell populations likely have opposing effects on hedonic feeding.

Fewer publications have investigated the role of the female rodent IL in reward seeking and motivational behaviors. To date, evidence suggests that the female IL may have more limited involvement in reward processing and positive affect. For instance, optogenetic activation of the glutamatergic IL to nucleus accumbens shell (NAcSh) pathway following conditioned taste aversion reduces aversive taste reactivity in males but not females. However, both sexes lever press for IL-NAcSh stimulation and a prior history of IL-NAcSh stimulation increases sucrose preference in males and females (Hurley and Carelli, 2020). Further, optogenetic stimulation of IL glutamatergic neurons induces a real-time place preference in males without affecting place preference or aversion in females, suggesting a positive valence to IL glutamatergic activity in males but not females (Wallace et al., 2021). These studies collectively indicate that, in males, IL activity is necessary for contextual appraisal during reward acquisition and that glutamatergic activity has positive valence. In contrast, current evidence suggests that female IL glutamatergic activity does not affect place preference or conditioned aversion, although activity in specific projections may be rewarding.

Table 2: Positively valenced stimuli. IL effects on reward behavior.

Reference	Sex	Species	IL Manipulation	Timing	Reward	Outcome
Eddy et al. 2016	Male	Wistar	Baclofen/muscimol	30-45 min before testing	Food pellet	Response in rewarding condition ↓ Response in extinct condition ↑
Capuzzo and Floresco 2020	Male	Long-Evans	Baclofen/muscimol	10 min before testing	Sucrose pellet	Inhibitory trial success ↓
Moorman and Aston-Jones 2015	Male	Sprague-Dawley	Electrical recording Baclofen/muscimol	Immediately prior	Sucrose	Recording: IL neural activity during sucrose acquisition ↑ Baclofen/muscimol: Lever press for reward ↓ Extinction ↓
Anthony Burgos-Robles et al. 2013	Male	Sprague-Dawley	Electrical recording Muscimol	30 min before testing	Sucrose pellet	Recording: IL neural activity during sucrose acquisition ↑ Muscimol: Reward collection latency ↑
Ferenczi et al. 2016	Male and Female	**Male:** Sprague-Dawley **Female:** TH-ChR2 rats	**Male:** CaMKII-SSFO: continuous, 4x over 6 hour testing **Female:** CaMKII-SSFO: continuous	During behavior	**Male:** Sucrose **Female:** Dopamine stimulation	**Males:** Sucrose preference ↓ **Females:** Preference for dopamine stimulation ↓
Newmyer et al. 2019	Male	VIP-Cre transgenic mice	Cre-dependent SSFO: continuous	5 min before testing	Palatable high-calorie diet	Palatable food intake ↓
Hurley and Carelli 2020	Male and Female	Sprague Dawley	IL-NAcSh CamKII-ChR2 stimulation: 5 s, 20 Hz, 10 mW	During behavior	Sucrose and stimulation	**Males but not females:** Aversive taste response ↓ **Both sexes:** Respond for stimulation ↑
Wallace et al. 2021	Male and Female	Spraque-Dawley	CaMKII-ChR2 stimulation: 5 ms, 10 Hz, 3 mW	During behavior	Real-time place preference	**Males:** Time in stimulation side ↑ **Females:** No preference

Social behavior

Reduced sociability is a common symptom of mood disorders. Additionally, decreased motivation for social interaction further worsens the course of depressive illness (Kupferberg et al. 2016). Consequently, determining how neural circuits encode and regulate social behavior is a critical area for investigation. Growing evidence indicates that male IL neural output regulates

the affective and motivational processes that underly social interaction (Table 3), possibly through descending limbic integration (Vertes, 2004; Wood et al., 2018). Indeed, pharmacological inactivation of the IL reduces both the frequency and duration of social play in adolescent male rats (Van Kerkhof et al., 2013). Furthermore, Minami et al. conducted electrical recordings of IL activity during social behavior and found that male IL neurons increase firing during the termination of social behavior, an effect absent in isolation-reared rats suggesting experience-dependent social encoding (Minami et al., 2017). SSFO enhancement of male IL glutamatergic neuron excitability reduces social interaction with a juvenile interactor (Ferenczi et al., 2016). In contrast, acute optogenetic stimulation of male IL glutamate neurons increases conspecific social motivation (Wallace et al., 2021), providing further evidence for contextual factors impacting IL-mediated behaviors. Increasing the excitability of male IL GABAergic VIP interneurons reduces novel social investigation, as well as novel object interactions (Newmyer et al., 2019). Other interneuronal investigations examined GABAergic parvalbumin (PV) neurons in the male and female mouse mPFC and found that PV neural activity increases in both sexes during social interactions compared to novel object interactions, a phenotype missing in the CNTNAP2 knockout autism model. Further, SSFO-increased excitability of PV interneurons rescues social deficits in the CNTNAP2 model, without impacting sociability in wildtype mice (Selimbeyoglu et al., 2017). Ultimately, these studies highlight the need to further investigate how specific interneuronal populations within the mPFC differentially encode and modulate social behavior.

Currently, evidence suggests the female IL may have a different role in social behavior. Optogenetic stimulation of IL glutamatergic neurons does not alter female social motivation or social novelty prefrence (Wallace et al., 2021). Further, the female IL appears to be less responsive to social interaction with conspecifics as female rats have less c-Fos expression compared to males after social interaction (Mikosz et al., 2015). Moreover, male rats have greater c-Fos responses to a previously-stressed interactor than an unstressed conspecific, an effect that does not occur in females. This sex difference may be independent of ovarian hormones as both intact cycling females and ovariectomized (OVX) females have similar IL c-Fos following social interaction (Mikosz et al., 2015). However, specific projection-defined IL neurons are necessary for social motivation. Huang et al. chemogenetically inactivated female basolateral amygdala-projecting IL neurons and abolished social preference in a 3-chamber social test (Huang et al., 2020). Overall, the data suggest that the male IL is more responsive to conspecific social interaction and that increasing male IL glutamatergic activity can bidirectionally modulate social motivation, dependent upon the method of stimulation, while interneuron stimulation produces opposing effects. Additionally, the female IL has less neural activity than males after conspecific interaction and stimulation of female IL glutamate neurons does not alter sociability. Although, amygdala-projecting female IL neurons are necessary for social preference.

While the female IL may be less involved in conspecific interaction, Pereira et al. 2020 demonstrated that the IL plays a critical role in maternal behaviors. Using a conditioned place preference paradigm, new mother rats spend equivalent time in chambers associated with cocaine reward and pups. However, blockade of sodium conductance in the female IL leads to an exclusive preference for the cocaine-paired chamber. Furthermore, IL inactivation reduces

maternal behaviors including nest building and retrievals. In fact, none of the IL-inactivated mothers fully retrieved all pups, while all vehicle-treated females did (Pereira and Morrell, 2020). Further, histological evidence indicates that the female IL shows greater activation following exposure to newborns than juvenile pups (Pose et al., 2019). Thus, the female IL may be more tuned to facilitate pup rearing than conspecific social interactions.

Table 3: Social behavior. IL effects on social behavior.

Reference	Sex	Species	IL Manipulation	Timing	Test	Outcome
Kerkhof et al. 2013	Male	Wistar	Baclofen/muscimol	5 min before testing	Free interaction	Social play ↓
Minami et al. 2017	Adolescent Male	Spraque-Dawley	Electrical recordings	During behavior	Free interaction	IL neural activity during interaction termination ↑
Ferenczi et al. 2016	Male	Sprague-Dawley	CaMKII-SSFO: continuous, 4x during testing	2 days prior and on testing day	Male juveline in homecage	Social interaction ↓
Selimbeyoglu et al. 2017	Male and Female	PV-Cre C57BL/6J	Border of IL and PL GCaMP6f photometry	During behavior	Free interaction	**Both sexes:** PV neural activity during social interaction ↑
Newmyer et al. 2019	Male	VIP-Cre transgenic	Cre-dependent SSFO: continuous	5 min before testing	Conspecific in homecage	Social interaction ↓
Wallace et al. 2021	Male and Female	Spraque-Dawley	CaMKII-ChR2 stimulation: 5 ms, 10 Hz, 3 mW	During behavior	3-chambered social test	**Males:** Social motivation ↑ **Females:** No change
Huang et al. 2020	Female	C57BL/6J	Cre-dependent inhibitory DREADD hM4Di in IL CAV2-Cre in BLA	CNO 30 min before testing	3-chambered social test	Social preference ↓
Pereira and Morrel 2020	Female	Sprague Dawley	Bupivacaine hydrochloride	5 min before testing	Pup-associated conditioned preference	Time in pup-associated zone ↓ Pup retrieval ↓

Gonadal hormone influences

The impact of gonadal hormones on neural activity may contribute to sexually divergent IL function. Gonadal hormones influence circuit regulation through both organizational effects in development as well as activational effects in adulthood. The importance of gonadal hormones for mPFC development, synapse formation, and pruning has been recently reviewed (Premachandran et al., 2020; Delevich et al., 2021). In adult rodents, estrogen receptors α and β (ERα and ERβ), are present in the male rodent mPFC (**Figure. 1**), distributed broadly across cortical layers in both pyramidal and non-pyramidal putative interneurons (Montague et al., 2008). Further, ultrastructural analysis found ERα, ERβ, and G protein-coupled estrogen receptor 1 (GPER1) in the female mPFC. Interestingly, GPER1 is expressed at over twice the levels of ERα and ERβ, suggesting a significant fast-acting component to ER signaling (Almey et al., 2014). Moreover, ER localization is largely extranuclear, with most receptors located axonally (Almey et al., 2014). Growing evidence suggests that ER signaling plays an important role in mPFC regulation of female behavior. For instance, 17β-estradiol (E2) localized to the female PL/IL junction shifts the cognitive strategy used for maze navigation (Almey et al., 2014). Further, ERα and ERβ agonist treatment in diestrus females potentiates the antidepressant-like effect of ketamine (Dossat et al., 2018). Recent evidence from OVX females also indicates that E2 increases the excitability of IL pyramidal neurons in slice and enhances extinction of reward-seeking (Yousuf et al., 2019). While more research is needed to determine the mechanisms by which estrogens regulate prefrontal function, these studies suggest that cyclic fluctuations in intrinsic network activity impact depression-related behaviors. Considerably less is known about the effects of cortical progesterone signaling. The female rodent frontal cortex expresses both progesterone receptor a (PRa) and b (PRb), with PRb levels decreasing during estrus (Guerra-Araiza et al., 2003). Although there are no reports, to our knowledge, of PR expression in males,

repeated progesterone administration increases $GABA_A$ receptor subunit α1 expression in the mPFC of both sexes (Andrade et al., 2012). Thus, cyclic increases in progesterone likely affect mPFC E/I balance.

Androgens may also play a role in IL functional differences as androgen receptors (AR) are expressed in the frontal cortex of male and female rodents (DonCarlos et al., 2006). Expression is higher in males than females and current evidence indicates little to no astrocyte expression, though this could be age-dependent (Feng et al., 2010). Further, AR expression in midbrain-projecting neurons suggests putative pyramidal expression (Aubele and Kritzer, 2012; Low et al., 2017). In addition, androgens regulate dopamine (DA) inputs to the male rodent mPFC. Orchiectomy increases DA axonal density and extracellular DA levels within the mPFC, an effect reversed by testosterone administration (Kritzer, 2003; Aubele and Kritzer, 2012). Further, a large portion of ventral tegmental area-projecting IL neurons express AR. Taken together, these results suggest a bidirectional interaction between androgen signaling and mesocortical DA circuitry that may influence IL network excitability as well as mood and behavior. Further evidence for gonadal hormone regulation of mPFC activity comes from studies indicating that androgens and estrogens have opposing effects on mPFC metabolism of DA, norepinephrine, and serotonin during a novel environment stressor (Handa et al., 1997). Collectively, this evidence suggests gonadal hormones modify IL function. Due to the widespread expression of these receptors in mPFC cell populations and varied actions on neural activity, considerable work remains to understand how hormonal fluctuations across the lifespan impact prefrontal network function.

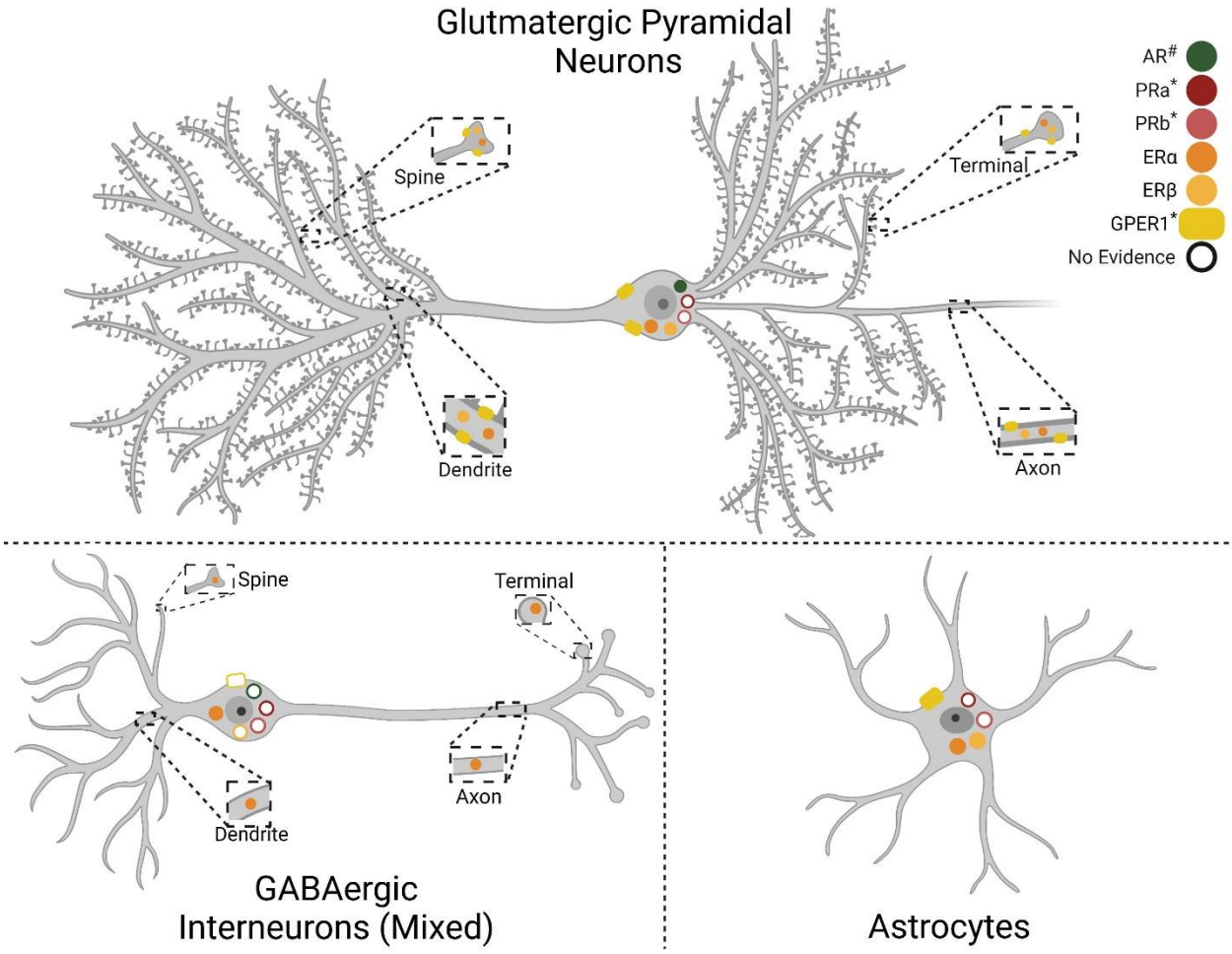

Figure 1 Gonadal hormone receptor expression in infralimbic cortex (IL) cell types. Depiction of gonadal hormone receptor expression and localization. Filled symbols indicate evidence for expression in the cell type and cellular compartment. Empty symbols denote lack of information as to the presence or absence of the receptor. Progesterone receptor cell-type specificity has not been reported. Androgen Receptor (AR), Progesterone Receptor A (PRa), Progesterone Receptor B (PRb), Estrogen Receptor α (ERα), Estrogen Receptor β (ERβ), G protein-coupled estrogen receptor 1 (GPER1). #AR expression is higher in males than females. *PRa, PRb, and GPER1 expression has only been reported in females.

Chronic stress impacts

The two greatest predictors of depressive outcomes are cumulative lifetime traumas and severe life stressors (Cassileth et al. 1984), indicating that the neural consequences of repeated or severe stress dictate disease burden. Mood-related symptoms, including negative affect,

anhedonia, despair, and social withdrawal, are also frequently initiated and/or exacerbated by prolonged stress (Kennedy and Adolphs 2012, Lupien et al. 2009). Accordingly, chronic stress exposure has been a primary preclinical paradigm for studying depression and mood disorders in animal models. In recent years, there has been growing interest in the sexual basis of chronic stress impacts on limbic structures. Multiple excellent reviews have covered the topic in-depth (McLaughlin et al., 2009; Bourke et al., 2012; Shansky and Woolley, 2016; Shors, 2016; Shepard and Coutellier, 2018; Fogaça and Duman, 2019; Moench et al., 2019; Page and Coutellier, 2019). Here, we review IL-specific effects.

Chronic stress-induced IL pyramidal neuron dendritic hypotrophy has been consistently reported in male rodents, though this varies based on projection targets (Cerqueira et al., 2005; Goldwater et al., 2009; Shansky et al., 2009; Luczynski et al., 2015; Czéh et al., 2018). Further, measurement of the long-term activation marker ΔFosB indicates the male IL is responsive to chronic stress exposure, an effect not present in other frontal regions such as the anterior cingulate or orbital cortices (Flak et al., 2012; Pace et al., 2020). However, studies of chronic stress effects on male IL glutamatergic excitability have yielded mixed results, contributing to opposing hypotheses of either hyper- or hypo-inhibition. McKlveen et al. (2016) found that IL pyramidal neurons of male rats exposed to a two-week variable stress paradigm had increased inhibitory currents and more GABAergic synaptic appositions, suggesting increased inhibition of IL glutamatergic neurons. GR was also reduced specifically in PV interneurons (McKlveen et al., 2016), pointing to the importance of glucocorticoid feedback for regulating local inhibition. In support of hyper-inhibition, chemogenetic inhibition of male mouse IL PV interneurons during CVS reduces passive coping in FST (Nawreen et al., 2020). Furthermore, chronic stress increases GAD67 mRNA in the male mouse IL (Shepard et al., 2016). Additionally, three weeks

of daily restraint stress in male mice increases dendritic arborization of GAD67-positive interneurons but reduces GAD67-positive somas (Gilabert-Juan et al.). In support of hypo-inhibition, Czek et al. 2018 found reductions in both IL interneuron populations and inhibitory currents in an anhedonic subpopulation of male rats exposed to nine weeks of variable stress (Czéh et al., 2018). Similarly, three weeks of chronic unpredictable stress reduces GAD67 mRNA in male rats, although vGluT1 mRNA is also reduced (Ghosal et al., 2020). Thus, there are data to support both increased and decreased inhibition of male IL pyramidal neurons after chronic stress. Multiple factors including differences in methodology, stress paradigms, and temporal factors may contribute to the discrepant findings. Further, how these post-mortem changes affect neural network activity and behavioral outcomes remains to be determined. In vivo, electrophysiology studies indicate male IL neurons increase firing during a shock-predicting cue; however, this effect is not present after repeated stress (Wilber et al., 2011). Taken together, chronic stress reduces male IL glutamatergic dendritic complexity and spine density, yet the chronic stress effects on inhibitory neural populations are mixed and have yet to reach a consensus.

Numerous female studies suggest that estrogen may be protective against chronic stress effects on IL function. Though not specific to the IL, Wei et al. (2013) found that repeated restraint stress reduced mPFC miniature excitatory postsynaptic current (mEPSC) amplitude and frequency in males but not females. The decreased excitability was accompanied by a male-specific reduction in glutamate receptor surface proteins. However, ER antagonism in females unmasked stress effects on mEPSC frequency and glutamate receptors, suggesting ER prevents excitatory hypofunction following stress. Intriguingly, estrogen delivery in males is sufficient to prevent stress effects on mEPSC amplitude and frequency, as well as partially restore glutamate

receptor surface protein expression (Wei et al., 2014). Moreover, female IL neurons generally do not show stress-induced dendritic remodeling. However, female IL pyramidal neurons projecting to the basolateral amygdala have estrogen-dependent increases in dendritic branching after repeated restraint stress (Shansky et al., 2010). Further, repeated stress increases spine density in this projection regardless of estrogen treatment (Shansky et al., 2010). Others have reported sex-specific effects of chronic variable stress based on IL projection target. Here, chronically-stressed female mice have increased EPSCs in the IL-NAc projection, while males have greater loss of dendritic complexity in VTA-projecting IL neurons. Additionally, chemogenetic inhibition of NAc-projecting IL neurons rescues chronic stress-induced behaviors only in females (Bittar et al., 2021). In contrast to pyramidal cells, PV interneurons appear to be more stress susceptible in females than males. Female IL PV mRNA increases following two weeks of daily stress exposure, with a further increase at four weeks. Females at four weeks also have increased PV neuron density and reduced IL c-Fos expression following open-field, effects that are absent in males (Shepard et al., 2016). However, both male and female mice have increased c-Fos in PV cells (Page et al., 2019), indicating increased interneuron activity. Although, chemogenetic activation of IL PV interneurons induces anxiety-like behavior only in females. Overall, these results indicate that estrogen is protective for female IL glutamatergic neurons, sex differences in chronic stress effects are projection-dependent, and interneuron populations are more susceptible to chronic stress in females.

Conclusion

The increased attention on females in preclinical research and the rapid development of neurobiological techniques with enhanced genetic and temporal specificity have isolated sex-specific regulatory roles of IL neural populations. Manipulations that induce long-term changes

in pyramidal E/I balance (SSFO-mediated hyperexcitability or lentiviral knockdown of glutamate release) can lead to divergent and sometimes contradictory behavioral outcomes. However, acutely increasing activity of IL glutamate neurons regulates numerous stress coping, motivational, and social behaviors in male rodents. The aggregate data suggest these cells promote active coping, context-appropriate reward-seeking, motivation, and sociability. Although, specific projections may have differing or even opposing actions. Less work has examined stress coping in females, but IL glutamate neurons have sexually divergent effects on physiological stress responses. Female IL pyramidal neurons may also play a smaller role in reward-seeking and motivational behaviors. In terms of sociability, the female IL seems less involved in conspecific interactions, with significant involvement in maternal behaviors. Many of these differences may be mediated by gonadal hormone signaling in different components of the IL neural and glial network. Generally, estrogens seem to protect glutamate neurons from the effects of chronic stress while androgens modulate cortical dopamine function. The sex-specific functions of the numerous IL interneuron subtypes remain to be determined. Further, the effects of chronic stress on IL cellular excitability are mixed for both sexes. Ultimately, complex interactions between sex and stress impact many aspects of vmPFC local networks and, consequently, brain-wide synaptic signaling. Determining the mechanistic basis of E/I balance in these cell groups is likely to significantly push forward our understanding of mood disorders and identify sex-specific treatment options to improve health outcomes.

Dissertation scope

As described in the introduction, the biological mechanisms underpinning mood-disorder symptoms remain unknown. Critically missing from our understanding is how specific neural populations within depression-associated regions regulate and respond to behaviors associated

with depression. To bridge this gap, the presented research focuses on the infralimbic cortex, IL, as the anatomical homologue of the human BA25. In Chapter 2, I utilize an optogenetic technique to increase the activity of IL glutamatergic neurons during behavioral and physiological testing. I conclude that there is considerable sexual divergence in IL glutamatergic neural regulation of affect, social behavior, and stress responding. In Chapter 3, I shift our stimulation to a hypothalamic region targeted heavily by IL projections, the posterior hypothalamus (PH). I perform similar testing to chapter 2, to determine what aspects of IL regulation are through PH directed signaling. Chapter 4 examines how IL glutamatergic neurons endogenously respond to behavioral states through fluorescent calcium signaling in awake behaving animals with and without a history of chronic stress. I continue to investigate sex differences in IL glutamatergic activity and examine how these differences in activity could be accounted for by ovarian hormones. Finally, Chapter 5 concludes with a discussion of how these findings fit into the broader literature and a more general discussion of behavioral neuroscience methodology.

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CHAPTER 2: ACUTE STIMULATION OF INFRALIMBIC GLUTAMATERGIC NEURONS HAS SEXUALLY DIVERGENT OUTCOMES ON MOOD DISORDER-RELATED BEHAVIORS.

Preamble

The following material was published April 28th, 2021, in Psychoneuroendocrinology under the title "Sexually divergent cortical control of affective-autonomic integration". Sections of results and materials referring to work included in another dissertation have been removed. References to we and our refer to co-authors on the published work.

Summary

Depression and cardiovascular disease reduce quality of life and increase mortality risk. These conditions commonly co-occur with sex-based differences in incidence and severity. However, the biological mechanisms linking the disorders are poorly understood. In the current study, we hypothesized that the infralimbic (IL) prefrontal cortex integrates mood-related behaviors with the cardiovascular burden of chronic stress. In a rodent model, we utilized optogenetics during behavior and *in vivo* physiological monitoring to examine how the IL regulates affect, social motivation, and neuroendocrine-autonomic stress reactivity. Our results indicate that IL glutamate neurons increase socio-motivational behaviors specifically in males. IL activation also reduced endocrine and autonomic stress responses in males, while increasing reactivity in females. Our findings suggest that cortical regulation of behavior and physiological stress responses fundamentally differ between sexes.

Background

Major depressive disorder (MDD) and cardio-metabolic conditions including hypertension, glucose intolerance, and heart failure significantly contribute to global disease burden. Epidemiological evidence implicates life stressors as a risk factor for both MDD and cardiovascular disease (CVD) (Grippo and Johnson, 2009; Binder and Nemeroff, 2010; Myers et al., 2014b; Sgoifo et al., 2015). Furthermore, sex differences in the incidence of MDD, CVD, and MDD-CVD co-morbidity suggest that sex-specific factors contribute to outcomes (Goldstein et al., 2019). However, the biological basis for stress effects on health, particularly the integration of affective and physiological systems, is poorly understood. Human brain imaging studies indicate that ventral medial prefrontal cortex (vmPFC) activity associates with sadness and blood pressure reactivity, suggesting that top-down cortical control may integrate diverse aspects of mood and systemic physiology.

The vmPFC is involved in numerous cognitive and emotional processes (Wood and Grafman, 2003; Myers-Schulz and Koenigs, 2012; McKlveen et al., 2015). A subregion of the vmPFC, the subgenual cingulate cortex (BA25), is activated by sadness-provoking stimuli, responds to social isolation, and has reduced volume in MDD patients (Liotti et al., 2000; Beckmann et al., 2009; Vijayakumar et al., 2017). BA25 is also targeted for deep brain stimulation in patients with treatment-resistant depression, where larger volumes predict better treatment outcomes (Mayberg et al., 2005; Sankar et al., 2019). Although, broader investigation of BA25 activity in mood disorders has yielded mixed results with reports of both hyper- (Mayberg et al., 2005; Hamani et al., 2011) and hypo-activity (Drevets et al., 1997, 2008). Subgenual regions of vmPFC have also been identified as components of a central autonomic network monitoring visceral functions (Gianaros and Sheu, 2009; Beissner et al., 2013; Gianaros and Wager, 2015; Myers, 2016; Shoemaker et al., 2016). Furthermore, recent pharmacological

studies employing glutamate uptake inhibitors in non-human primates have implicated BA25 activity in reduced reward motivation (Alexander et al., 2019) and enhanced threat-related autonomic responses (Alexander et al., 2020). The rodent putative anatomical homolog of BA25, the infralimbic cortex (IL) (Öngür et al., 2003; Uylings et al., 2003; Vertes, 2004; Roberts and Clarke, 2019), innervates limbic and stress-regulatory nuclei including the amygdala, thalamus, and hypothalamus (Gabbott et al., 2005; Myers et al., 2014a, 2016; Wood et al., 2018). Additionally, IL stimulation reduces passive coping and increases pyramidal neuron spine density in male rodents (Fuchikami et al., 2015). Moreover, knockdown of IL glutamatergic output exacerbates chronic stress effects on hypothalamic-pituitary-adrenal (HPA) axis reactivity and vascular function (Myers et al., 2017; Schaeuble et al., 2019). However, the potential sex-specific roles of IL activity to integrate socio-motivational behaviors with physiological stress reactivity remain to be determined.

To identify how the male and female vmPFC coordinates mood-related behaviors and cardiovascular outcomes, genetically identified vmPFC projection neurons received temporally-specific stimulation in an *in vivo* rodent model. Specifically, channelrhodopsin-2 (ChR2) was expressed under the calcium/calmodulin-dependent protein kinase type II α (CaMKIIα) promoter in the IL to permit optogenetic activation of IL pyramidal neurons in both male and female rats (Wood et al., 2018). This approach was combined with measures of place preference and social behavior to examine affective valence and sociability. Behavioral assessment was followed by measures of physiological stress reactivity. Ultimately, these findings identify the IL as an affective-autonomic integrator that links motivation and stress responding divergently in males and females.

Methods

Animals

Age-matched adult male and female Sprague-Dawley rats were obtained from Envigo (Denver, CO) with male rat weight ranging from 250-300 g and female from 150-200 g. After stereotaxic surgery, rats were housed individually in shoebox cages with cardboard tubes for enrichment in a temperature- and humidity-controlled room with a 12-hour light-dark cycle (lights on at 07:00h, off at 19:00h) and food and water *ad libitum*. In accordance with ARRIVE guidelines, all treatments were randomized and experimenters blinded. All procedures and protocols were approved by the Colorado State University Institutional Animal Care and Use Committee (protocol: 16-6871A) and complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Signs of poor health and/or weight loss ≥ 20% of pre-surgical weight were *a priori* exclusion criteria. These criteria were not met by any animals in the current experiments; however, animals were removed from experimentation if fiber optics failed.

Microinjections

Rats were anesthetized with isoflurane (1-5%) and administered analgesic (0.6 mg/kg buprenorphine-SR, subcutaneous). Rats received bilateral microinjections (Males 1.5 - 2 µL, Females 0.75 – 1.25 µL) of adeno-associated virus (AAV) into the IL (males: 2.7 mm anterior to bregma, 0.6 mm lateral to midline, and 4.2 mm ventral from dura, females: 2.3 mm anterior to bregma, 0.5 mm lateral to midline, and 4 mm ventral from dura). These volumes correspond with prior studies utilizing viral vector transduction in rat vmPFC (Ji and Neugebauer, 2012; Ferenczi et al., 2016; Wood et al., 2018). AAV5-packaged constructs (University of North Carolina Vector Core, Chapel Hill, NC) either expressed yellow fluorescent protein (YFP) or ChR2

conjugated to YFP under the CaMKIIα promoter to achieve pyramidal cell-predominant expression (Wood et al., 2018). All microinjections were carried out with a 25-gauge, 2-µL microsyringe (Hamilton, Reno, NV) using a microinjection unit (Kopf, Tujunga, CA) at a rate of 5 minutes/µL. The needle was left in place for 5 minutes before and after injections to reduce tissue damage and allow diffusion. Skin was closed with wound clips that were removed 2 weeks after injections and animals were allowed at least 6 weeks for recovery and ChR2 expression.

Electrophysiology

Adult male rats (n = 8) were injected with AAV constructs as described above and, after 8-12 weeks, exposed to 5% isoflurane prior to decapitation and brain removal. As previously described (Rau and Hentges, 2017), brains and sections were collected in ice-cold artificial CSF (aCSF) consisting of the following (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$ Λ $6H_2O$, 2.4 $CaCl_2$ Λ $2H_2O$, 1.2 NaH_2PO_4, 11.1 glucose, and 21.4 $NaHCO_3$, bubbled with 95% O_2 and 5% CO_2. Coronal slices containing the IL were cut at a thickness of 240 □m using a model VT1200S vibratome (Leica Microsystems, Buffalo Grove, IL). After resting 1 hr at 37°C in aCSF, slices were transferred to the recording chamber and perfused with oxygenated 37° C aCSF at a 2 ml/min flow rate. For whole-cell recordings, the internal recording solution contained the following (in mM): KCL 57.5, K-methyl sulfate 57.5, NaCl 20, $MgCl_2$ 1.5, HEPES 5; EGTA 0.1; ATP 2; GTP 0.5, and phosphocreatine 10. The pH was adjusted to 7.3. Recording electrodes had a resistance of 2 – 4 MΩ when filled with this solution. IL pyramidal neurons were identified for recording based on the expression of ChR2-YFP under the control of CaMKIIα. Whole-cell patch-clamp recordings were acquired in voltage-clamp at a holding potential of -60 mV using an Axopatch 200B Amplifier (Molecular Devices, San Jose, CA). Current-clamp recordings

were acquired while holding current at 0 pA. Electrophysiological data were collected and analyzed using Axograph X software on a Mac OS X operating system (Apple, Cupertino, CA). Light activation of IL neurons expressing ChR2 occurred via 473 nm LED (Thorlabs, Newton, NJ) 1.1 mW light pulse driven by a LEDD1B driver (Thorlabs, Newton, NJ) triggered through the TTL output on an ITC-18 computer interface board (HEKA Instruments, Holliston, MA). Current-clamp experiments utilized 5, 10, and 20 Hz stimulation frequencies for 5 min bouts. Recordings were excluded if access resistance exceeded 10 Ω during recording.

Fiber optic cannulas

Rats were anesthetized with isoflurane (1-5%) followed by analgesic (0.6 mg/kg buprenorphine-SR, subcutaneous) and antibiotic (5 mg/kg gentamicin, intramuscular) administration. Bilateral fiber-optic cannulas (flat tip 400/430 μm, NA = 0.66, 1.1 mm pitch with 4.5 mm protrusion for males and 4.2 mm protrusion for females; Doric Lenses, Québec, Canada) were aligned with the IL injection sites and lowered to the ventral PL/dorsal IL approximately 1 mm dorsal to the injection to enable optic stimulation of the IL. Cannulas were secured to the skull with metal screws (Plastics One) and dental cement (Stoelting, Wood Dale, IL). Skin was sutured and, following 1 week of recovery, rats were handled daily and acclimated to the stimulation procedure for another week before experiments began. Rat handling and cannula habituation continued daily throughout experiments.

Optogenetic stimulation

Light pulses (1.0 - 1.3 mW, 5 ms pulses, 10 or 20 Hz) were delivered through a fiber-optic patch cord (240 μm core diameter, NA = 0.63; Doric Lenses) connected to a

473 nm LED driver (Doric Lenses). Optic power was measured with a photodiode sensor (PM160, Thorlabs Inc, Newton, NJ) at the cannula fiber tip. Initial male experiments used 20 Hz stimulation but, following the results of slice electrophysiology experiments, all subsequent studies used 10 Hz stimulation. Rats received a single 20-minute session of stimulation (1 min on/1 min off) in their homecage the week prior to stress exposure. In total, rats received self-determined stimulation in the stimulation zone of RTPP day 2, throughout the social behavior assay, and during acute stress exposure (restraint and novel environment). Further optic details are elaborated in the specified sections below.

Estrous cycle cytology

All female rats went through experiment 3 simultaneously, housed in the same room and randomly cycling. Immediately following acute measures of behavior and physiology, vaginal cytology was examined to approximate the estrous cycle stage. A damp (deionized water) cotton swab was used to collect cells from the vaginal canal and place them onto a glass slide. When dried, slides were viewed under a 10x objective light microscope by a minimum of two blind observers and were categorized as proestrus, estrus, metestrus, or diestrus (Smith et al.; Cora et al., 2015; Solomon et al., 2015).

Real-time place preference

The real-time place preference (RTPP) assay was used to assess the valence of IL stimulation (Stamatakis and Stuber, 2012; Bimpisidis et al., 2020). Cannulas were connected via patch cords to LEDs for light delivery and rats placed in a custom-made fiberglass arena with two chambers connected by a corridor (chambers: 15 x 15’’, corridor: 8 x 6’’, 15’’ deep). Rats

explored the arena for 10 minutes on two consecutive days. The first day was a habituation day and no stimulation was delivered on either side. On the second day, rats received LED-generated 470 nm light pulses upon entry and throughout the time spent in the assigned stimulation side. Stimulation stopped when rats exited the assigned stimulation side but re-commenced upon re-entry. Thus, rats determined the amount of stimulation received through time spent in the stimulation side. Trials were recorded by a camera mounted above the arena and animal position was tracked by Ethovision software (Noldus Information Technologies) for automated optic hardware control. Stimulation side assignment was counterbalanced, and animal testing was randomized. The time rats spent in the stimulation side was divided by the total time and multiplied by 100 to generate a percentage of time spent in the stimulation side.

Social behavior

A modified version of the 3-chambered social behavior assay was used to accommodate optic patch cords (Moy et al., 2004; Felix-Ortiz and Tye, 2014). To examine social interaction, each rat was connected to a patch cord and placed in a black rectangular fiberglass arena (36 x 23'', 15.8'' deep). Initially, the arena was empty and experimental rats were allowed to explore for 5 minutes without optic stimulation. The experimental rat was then returned to their home cage while an empty enclosure (ventilated with small round openings) was placed on one side of the arena, defined as the object, and an identical enclosure containing an age- and sex-matched conspecific was placed on the other side of the arena, defined as the social cage. The experimental rat was then placed in the middle of the arena and allowed to explore freely for 10 minutes with 5 ms pulsatile stimulation delivered throughout to quantify social motivation. The experimental rat was then placed again into its homecage while the empty enclosure was

replaced with a new enclosure containing a novel age- and sex-matched conspecific. The experimental rat freely explored for 10 minutes while receiving optic stimulation to assess social novelty preference. Behavior was recorded with an overhead camera and interactions were defined as nose pokes onto cages and scored by a treatment-blinded observer. The duration of interactions was divided by the total time of each interaction period and multiplied by 100 to give a percent interaction value. Sides for object cage, social cage, and novel cage were counterbalanced and animal order randomized.

Restraint stress

Restraint was used to examine neuroendocrine responses to acute stress. Rats were placed in plastic film decapicones (Braintree Scientific, Braintree, MA) and connected to fiber-optic patch cords for optic stimulation throughout the 30-minute restraint. Blood samples (approximately 250 μL) were collected by tail clip at the initiation of restraint with additional samples taken 15 and 30 min after (Vahl et al., 2005). At the conclusion of restraint, patch cords were disconnected, and rats returned to their homecage with recovery blood samples collected at 60 and 90 min after the initiation of restraint. Blood glucose was determined with Contour Next EZ glucometers (Bayer, Parsippany, NJ) and 2 independent readings for each time point were averaged. Blood samples were centrifuged at 3000 X g for 15 minutes at 4° C and plasma was stored at –20° C until radioimmunoassay (RIA). Plasma corticosterone levels were measured with an ^{125}I RIA kit (MP Biomedicals, Orangeburg, NY) as previously described (Myers et al., 2017). All samples for each sex were run in duplicate and all time points were run in the same assay. RIA intra-assay coefficient of variation was 8.6% and interassay 13.6%.

Tissue collection

At the conclusion of experiments, rats were given an overdose of sodium pentobarbital and perfused transcardially with 0.9% saline followed by 4.0% paraformaldehyde in 0.1 M PBS. Brains were removed and post-fixed in 4.0% paraformaldehyde for 24 h at room temperature, followed by storage in 30% sucrose in PBS at 4 °C. Coronal sections were made on a freezing microtome at 30 μm thickness and then stored in cryoprotectant solution at −20 °C until processing. A subset of rats received optogenetic stimulation prior to tissue collection. Rats were tethered to a fiber optic patch cord and received 5 minutes of optic stimulation (1 mW, 5 ms pulses, 10 Hz) followed by 90 minutes of recovery for immediate-early gene (c-Fos) expression prior to euthanasia, as described above.

Immunohistochemistry and microscopy

For fluorescent labeling of c-Fos, coronal brain sections were removed from cryoprotectant and rinsed in PBS (5 x 5 min) at room temperature. Sections were then placed in blocking solution (PBS, 0.1% bovine serum albumin, and 0.2% Triton X-100) for 1 hour. Next, sections were incubated overnight in rabbit anti-c-Fos primary antibody (1:200 in blocking solution, Cell Signaling Technologies, Ab #2250). Following overnight incubation in primary antibody, sections were rinsed in PBS (5 x 5 min) and incubated in donkey anti-rabbit Cy5 secondary (1:1000 in PBS, Jackson ImmunoResearch, AB_2340607) for 1 hour. The tissue was then washed (5 x 5 min in PBS), mounted with polyvinyl medium, and cover slipped for imaging. For chromogen labeling of c-Fos positive nuclei, sections were first rinsed in PBS (5 x 5 min) at room temperature. Sections were then incubated in 1% hydrogen peroxide in PBS for 10 minutes followed by a second PBS (5 x 5 min) wash. Sections were then placed in blocking

solution for 1 hour. Following blocking, sections were incubated overnight in rabbit anti-c-Fos primary antibody (1:2000 in blocking solution, Abcam, ab190289). Sections were then rinsed in PBS (5 x 5 min) followed by a 1-hour incubation in biotinylated goat anti-rabbit secondary (1:500 in PBS, Vector Laboratories, BA-1000). Sections were rinsed in PBS (5 x 5 min) followed by a 1-hour incubation in Vectastain ABC Solution (1:1,000; Vector Laboratories). Sections were rinsed again prior to incubation in diaminobenzidine and hydrogen peroxide (0.02% diaminobenzidine/0.09% hydrogen peroxide in KPBS) for 10 min. Following incubation, slices were rinsed, slide mounted, dehydrated in graded ethanol, and cover slipped.

To determine injection placement, YFP was imaged with a Zeiss Axio Imager Z2 microscope using the 10x objective, while YFP and c-Fos dual fluorescence were acquired as tiled 20x objective images. For quantification of c-Fos positive cells, slides were imaged in brightfield with the 10x objective.

Quantification of c-Fos

To quantify the number of c-Fos positive cells, 3-4 IL micrographs adjacent to visible cannula tracts were imaged and positive cells within a half-width 10x frame were hand-counted by a treatment-blind observer.

Experimental design

Timelines for experiments are outlined in figure 1. Experiment 1 was comprised of 2 cohorts of male rats (n = 13-14 each) to yield 10 YFP and 17 ChR2. For this experiment, real-time place preference and social behavior were assessed prior to restraint stress with 20 Hz used for all stimulation. A parallel group of injected male rats (n = 8) were used for slice

electrophysiology. Experiment 2 consisted of 3 cohorts of male rats (n = 7-8 each) for a total of 12 YFP and 10 ChR2. All animals underwent real-time place preference and social behavior prior to novel environment with 10 Hz stimulation. Experiment 3 was designed as a single cohort of female rats (n = 29) with 12 YFP and 17 ChR2. Real-time place preference and social motivation were followed by novel environment and restraint with 10 Hz stimulation for all measures. A subset of rats in experiments 2 and 3 (n = 6-7/group/sex) received optic stimulation prior to tissue collection to verify neuronal activation.

Data analysis

Data are expressed as mean ± standard error of the mean. Data were analyzed using Prism 8 (GraphPad, San Diego, CA), with statistical significance set at $p < 0.05$ for rejection of null hypotheses. Stimulation induced c-Fos was analyzed with Welch's unpaired t-test comparing virus groups. RTPP stimulation preference was assessed via repeated measure two-way analysis of variance (ANOVA) with virus and day (repeated) as factors. In the case of significant main or interaction effects, Sidak's multiple comparisons were run to determine group differences. Social motivation and novelty preference were assessed with unpaired t-tests to compare virus groups within social, object, novel rat, or familiar rat interactions. Total distance traveled during RTPP and social interaction was assessed with unpaired t-tests comparing virus groups. Stress responses over time (corticosterone and glucose) were analyzed using mixed-effects analysis with virus and time (repeated) as factors, followed by Fisher's post hoc if significant main or interaction effects were present.

Results

Validation and design

AAV viral vectors were targeted to the IL for expression of membrane-targeted ChR2-YFP or cytosolic YFP under the pyramidal neuron promoter, CaMKIIα. Fiber optic cannulas implanted in the IL permitted selective stimulation of IL glutamatergic neurons (**Figure. S1**), as previously reported (Fuchikami et al., 2015; Wood et al., 2018). In female rats, representative c-Fos labeling demonstrated *in vivo* neural activation in response to light-stimulation (1 mW, 10 Hz, 5 ms, 5 min) (**Figure. 2A**). c-Fos quantification in both male and female rats (**Figure. S2**) further validated stimulation efficacy. Whole-cell patch-clamp recordings in slice demonstrated light-evoked depolarizing current in male IL neurons expressing ChR2 (**Figure. 2B**). Further, 10 Hz stimulation led to high-fidelity action potential generation. 20-Hz light pulses induced action potential firing, but also increased resting membrane potential (**Figure. 2C**). These results, combined with our previous studies quantifying increased immediate-early gene expression after 20 Hz stimulation (Wood et al., 2018), suggest that both 10 Hz and 20 Hz stimulation activate IL CaMKIIα-positive neurons. Although, 10 Hz stimulation is more in line with the reported 4-10 Hz intrinsic firing rate of IL pyramidal neurons (Homayoun and Moghaddam, 2007; Ji and Neugebauer, 2012). The experimental design (**Figure. 2D**) is detailed in the methods. Male and female rats received optogenetic stimulation during behavioral and physiological measures in separate experiments. At the conclusion of all experiments, the placement of viral injections and fiber optics was determined. Only animals with cannula placement verified to be within or immediately (within 0.5 mm) dorsal to the IL were included in analyses (**Figure. 2E**).

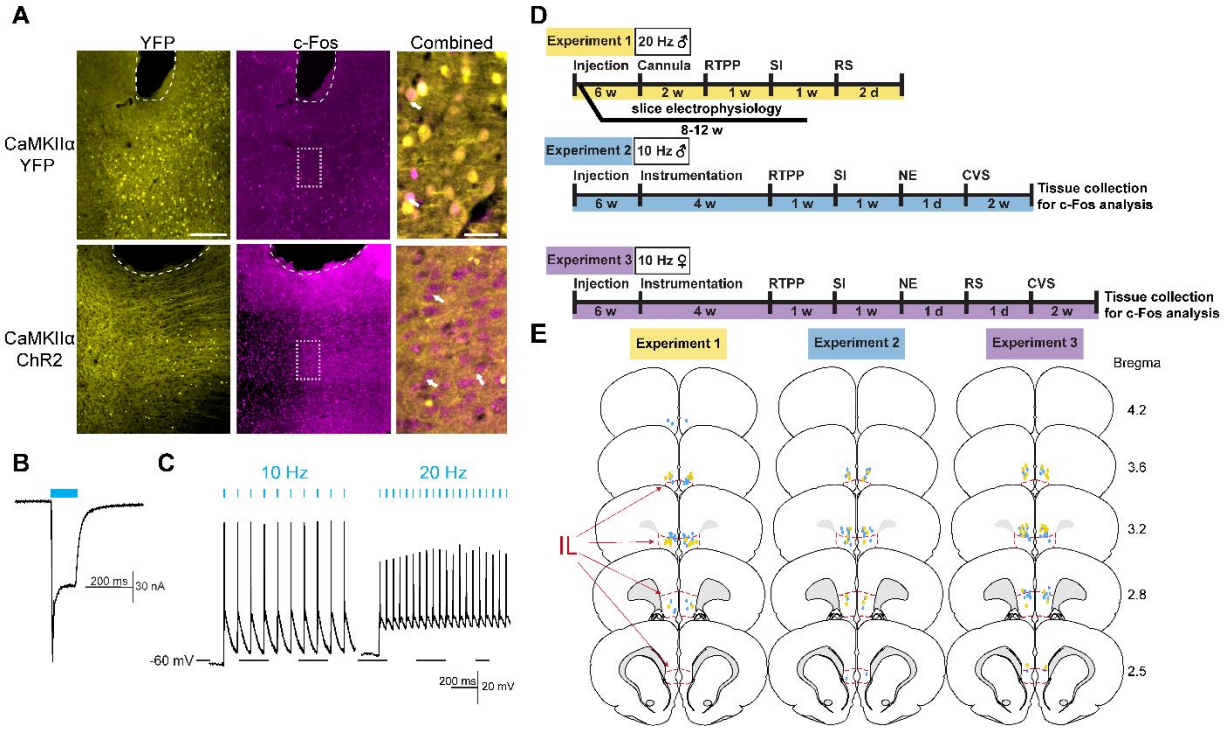

Figure 2: Approach validation and experimental design. (**A**) Injection of AAV-packaged constructs led to expression of cytosolic YFP or membrane-targeted ChR2-YFP under the CaMKIIα promoter. Blue light stimulation in ChR2 females (1 mW, 10 Hz, 5 ms pulses for 5 min) led to robust expression of the immediate-early gene marker c-Fos. Bregma +2.8mm. White arrows indicate representative c-Fos-positive nuclei. Dashed white lines indicate fiber tip. Scale bar: 200 µm and 40 µm for combined. (**B**) Voltage-clamp recordings from male IL-containing slices illustrated light-evoked (1.1 mW, 100 ms pulse) depolarizing current. (**C**) Current-clamp recordings found stimulation-locked spiking with 10 and 20 Hz stimulation (1.1 mW, 5 ms pulse). (**D**) Experimental timelines. RTPP: real-time place preference, SI: social interaction, RS: restraint stress, NE: novel environment, CVS: chronic variable stress. (**E**) Optic fiber placements (YFP: yellow, ChR2: blue) were mapped within or immediately dorsal to the IL (red outline). Coronal sections adapted from Swanson Rat Brain Atlas (3rd edition).

Real-time place preference

RTPP was used to examine the affective valence of stimulating IL glutamatergic neurons. Group mean heat maps illustrate time spent in the stimulation versus non-stimulation chambers (**Figure. 2A**). Male rats showed a preference for the stimulation chamber (**Figure. 2B**) with 20 Hz (repeated-measures 2-way ANOVA: stimulation x ChR2 $F_{(1, 23)} = 15.04$, $p < 0.001$)

and 10 Hz stimulation (repeated-measures 2-way ANOVA: stimulation $F_{(1, 20)} = 6.03$, $p < 0.05$, ChR2 $F_{(1, 20)} = 9.04$, $p < 0.01$, stimulation x ChR2 $F_{(1, 20)} = 6.43$, $p < 0.05$). Specifically, Sidak's *post hoc* indicated a preference for the 20 Hz and 10 Hz stimulation chambers compared to habituation day in the ChR2 groups (n = 10-15, $p < 0.01$). ChR2 groups also preferred the stimulation chamber relative to YFP animals on the stimulation day (n = 10-12, $p < 0.01$). In contrast, female rats demonstrated no preference or aversion for the chamber paired with IL stimulation (n = 11-13/group, repeated-measures 2-way ANOVA: stimulation $F_{(1, 22)} = 5.79$, $p < 0.05$, stimulation x ChR2 $F_{(1, 22)} = 0.005$, $p = 0.94$). Additionally, IL stimulation did not affect general locomotor activity in either sex (**Figure. 2C**). Together, these findings indicate that the activity of male IL glutamatergic neurons has a positive affective valance as animals were motivated to seek out the stimulation. However, this cell group does not appear to modify affective state in females.

Social behavior

The three-chamber social interaction assay was used to determine the influence of IL glutamatergic neurons on sociability. During the social motivation test (**Figure. 2D**), there was no difference in social interactions with 20 Hz stimulation in males (n = 9-10/group, unpaired t-test: ChR2 vs YFP $t_{(17)} = 0.77$, $p = 0.45$). However, 10 Hz stimulation in males increased social interactions (**Figure. 2E**; n = 10-12/group, unpaired t-test: ChR2 vs YFP $t_{(18)} = 3.00$, $p < 0.01$). In contrast, 10 Hz stimulation in females did not impact social interaction (n = 12-16/group, unpaired t-tests: ChR2 vs YFP $t_{(24)} = 1.01$, $p = 0.32$). Furthermore, neither male nor female IL stimulation affected preference for novel vs. familiar interactors (**Table S1**). Overall, these results indicate that IL glutamatergic activity increases male social motivation in a frequency-dependent manner; however, this cell population does not alter female social motivation.

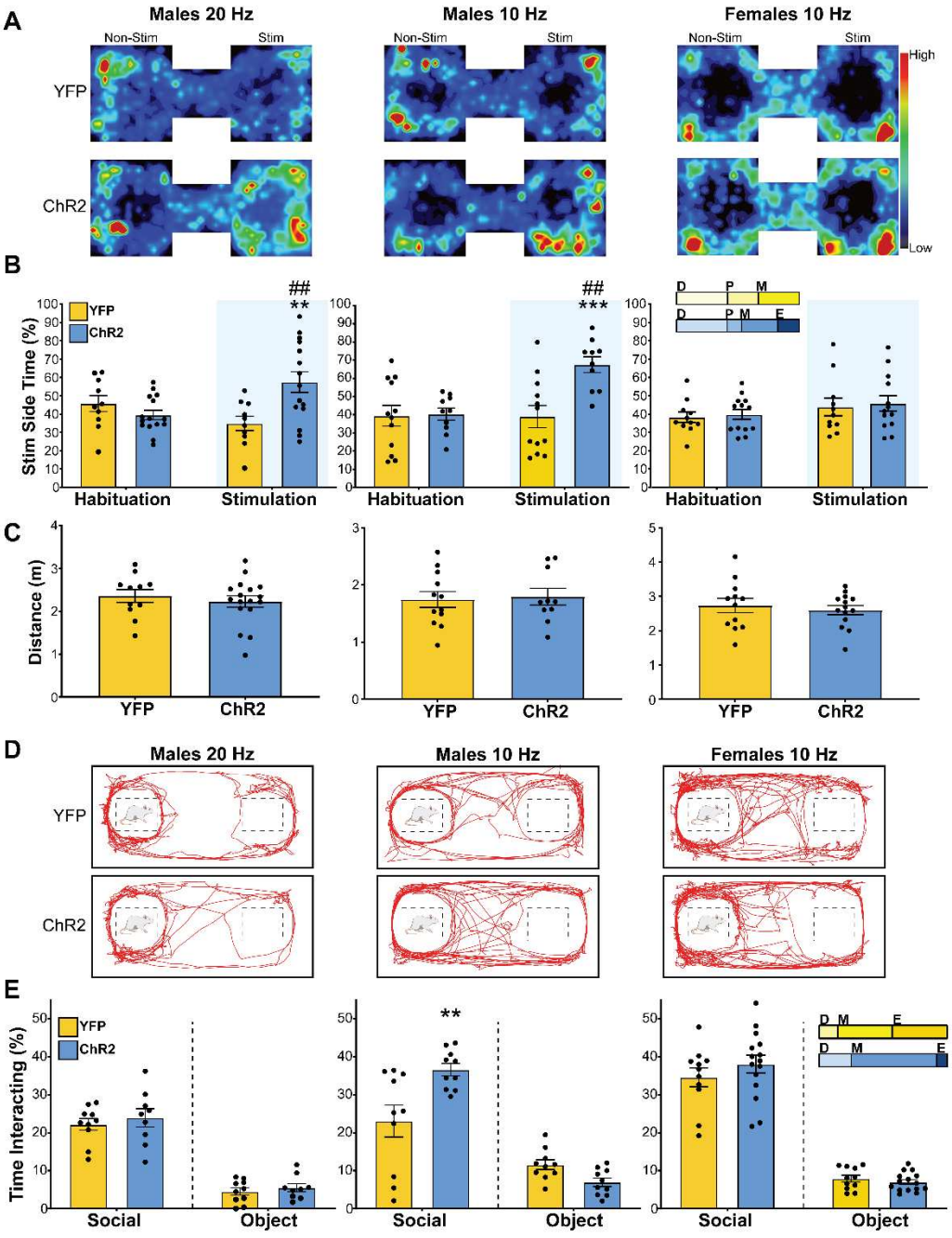

Figure 3: IL pyramidal neuron activity was preferred and increased social motivation in males but not females. **A**) Heat maps illustrate mean animal position in RTPP arena on stimulation day (n = 10-15/group). (**B**) Male ChR2 rats preferred the chamber paired with 20 Hz or 10 Hz stimulation (blue shading) relative to habituation day and YFP controls. Females showed no preference or aversion for IL stimulation. ** $p < 0.01$, *** $p < 0.001$ vs. YFP within stimulation. $^{\#\#}$ $p < 0.01$ vs. habituation within ChR2. Inset: portion of rats in each estrous cycle

phase. D: diestrus, P: proestrus, M: metestrus, E: estrous. (**C**) Total distance traveled during stimulation found no treatment-based differences in locomotion. (**D**) Representative movement traces during the social interaction test (n = 7-16/group). (**E**) Interaction, defined as nose-poke onto social cage, was increased by 10 Hz stimulation in males but not 20 Hz in males or 10 Hz in females. ** $p < 0.01$ vs. YFP. Inset: portion of rats in each estrous cycle phase.

Endocrine reactivity

To determine the effect of stimulating IL pyramidal neurons on neuroendocrine responses, blood glucose and plasma corticosterone were monitored during restraint stress. In males, optic stimulation decreased corticosterone (n = 10-17/group, mixed-effects: time $F_{(4,81)} = 139.4$, $p < 0.0001$, ChR2 $F_{(1,25)} = 8.93$, $p < 0.01$) at the 30-minute timepoint (**Figure. 3A**; $p < 0.01$). Additionally, corticosterone was decreased post-stimulation in the ChR2 group during stress recovery (90 min; $p < 0.05$). Stimulation also decreased blood glucose (mixed-effects: time $F_{(4,90)} = 62.86$, $p < 0.0001$, time x ChR2 $F_{(4,90)} = 3.43$, $p < 0.05$) in male rats during restraint (**Figure. 3B**; 15 min, $p < 0.01$; 30 min, $p < 0.05$). In contrast, stimulation did not alter plasma corticosterone in female rats (**Figure. 3C**; n = 10-17/group, mixed-effects: time $F_{(4,93)} = 62.94$, $p < 0.0001$) with effects of time limited to within treatment. Additionally, stimulation increased glucose responses to stress in female rats (n = 10-17/group, mixed effects: time $F_{(4,90)} = 24.96$, $p < 0.0001$) specifically at the 15-min timepoint (**Figure. 3D**; $p < 0.05$). Collectively, these results suggest that IL activity in males reduces HPA axis activation as well as glucose mobilization. In females, IL activity does not appear to alter HPA axis response to stress. However, IL stimulation in females increases glucose, possibly through sympathetic mobilization of epinephrine and/or glucagon.

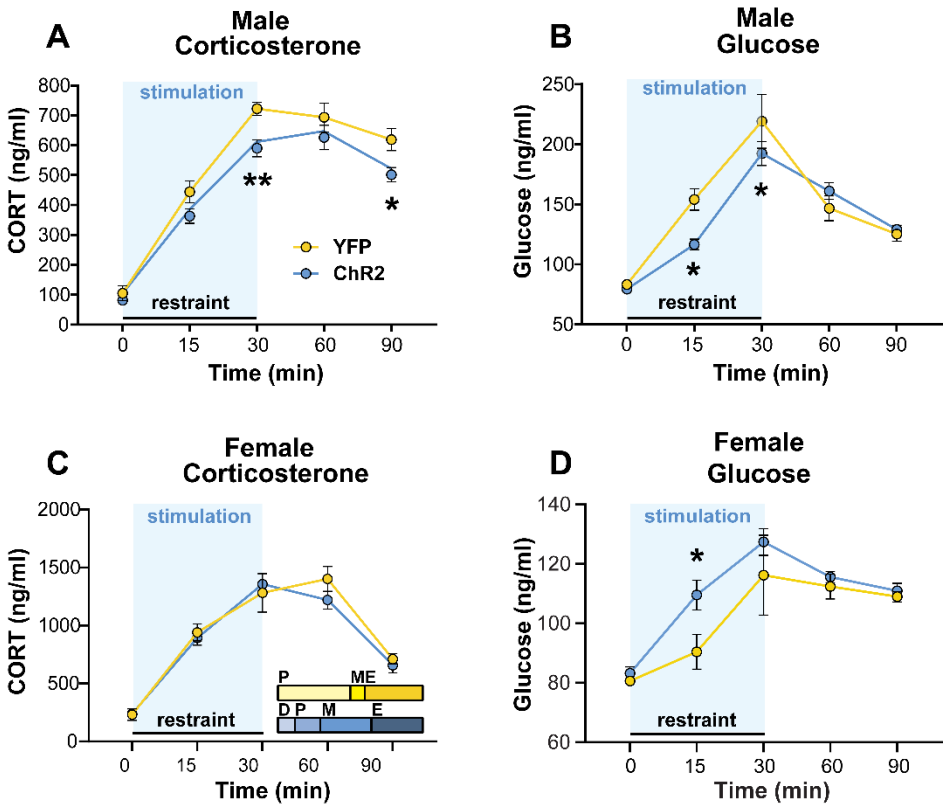

Figure 4: IL activation during acute restraint stress attenuated endocrine responses in males but increased female stress reactivity. Stimulation (blue shading) during restraint lowered plasma corticosterone **(A)** and blood glucose **(B)** in ChR2 males. **(C)** Stimulation in females did not alter corticosterone responses. Inset: portion of rats in each estrous cycle phase. D: diestrus, P: proestrus, M: metestrus, E: estrous. **(D)** IL stimulation during restraint increased glucose mobilization in ChR2 females. n = 10-17/group, * $p < 0.05$, ** $p < 0.01$ vs. YFP.

Discussion

In the current study, optogenetic stimulation of glutamatergic IL pyramidal neurons was combined with behavioral, endocrine, and autonomic assessments. Our results show that, in males, IL pyramidal neuron activity was preferred, increased social motivation, and reduced acute physiological stress reactivity. However, IL activity had fundamentally different regulatory effects in females. Stimulation did not have motivational valence or alter social behavior but increased acute physiological stress reactivity. Collectively, these findings identify sexual

divergence in the cortical integration of affective and physiological systems, suggesting that vmPFC output signaling may differentially impact health outcomes in males and females.

The comorbidity of CVD and MDD shows sexual divergence with females at twice the risk (Naqvi et al., 2005; Möller-Leimkühler, 2007; Goldstein et al., 2019). Given the interactions between stress, mood disorders, and CVD, stress-reactive neural populations are well positioned to regulate affective and cardiovascular outcomes. Importantly, chronic stress exposure in male rats reduces IL pyramidal neuron dendritic arborization and increases local GABAergic signaling, suggesting reduced glutamatergic output (Radley et al., 2008; McKlveen et al., 2016, 2019). Further, long-term reduction of male IL glutamatergic output increases HPA axis activity and impairs vascular function (Myers et al., 2017; Schaeuble et al., 2019). Collectively, IL output neurons represent a target for modulating behavioral and physiological responses to stress. Thus, we sought to test this hypothesis through real-time *in vivo* assessments of behavior and physiology. Altogether, we found that the neurobiology of emotional behaviors and endocrine-autonomic integration differed substantially between sexes.

The RTPP test was used to assess the affective state of experimental animals by measuring preference or aversion for IL neural activity. Here, male rats preferred the chamber paired with either 10 Hz or 20 Hz stimulation, but the valence of female IL activation was neither positive nor negative. Previous work indicates that male mPFC stimulation reduces social avoidance after social defeat (Covington et al., 2010), yet relatively few studies have examined IL function in females. Although social interaction induces less IL immediate early-gene expression in females than males (Stack et al., 2010; Mikosz et al., 2015), the current study is, to our knowledge, the first examination of IL regulation of female social behavior. IL activity did not alter social behavior in females, indicating a significant sexual divergence in the neural

regulation of sociability. However, males exhibited frequency-dependent increases in social motivation. These results suggest that stimulation near the intrinsic pyramidal neuron firing rate (4-10 Hz) (Homayoun and Moghaddam, 2007; Ji and Neugebauer, 2012) is required to increase male social motivation.

Activation of IL glutamatergic neurons also reduced male glucose and corticosterone responses to stress. Conversely, female IL glutamatergic stimulation increased glucose mobilization without affecting corticosterone, suggesting a role in sympatho-excitation. These experiments indicate that IL activity has opposing effects on endocrine-autonomic integration in males and females whereby male glutamatergic IL neurons cause widespread inhibition of the stress response and female IL neurons facilitate sympathetic responses.

Physiological responses to stress are critical for survival, tightly defended, and limited in maximum capacity; accordingly, our manipulations only caused moderate changes in response magnitude to acute challenges. However, our findings suggest that the interaction of altered stress responding with chronic stressors is sufficient to account for differences in cardiac structure and function. It is also worth noting that prior studies on the role of vmPFC in stress responding have yielded equivocal results. Specifically, lesions studies in male rats have observed both increased (Diorio et al., 1993; Figueiredo et al., 2003) and decreased (Radley et al., 2006) neuroendocrine stress responding. Similarly, pharmacological studies found that male vmPFC both reduces (Müller-Ribeiro et al., 2012) and enhances (Tavares et al., 2009) cardiovascular stress responses. These discrepancies are not limited to rodents as pharmacological studies in non-human primates suggest that vmPFC increases cardiovascular stress responding (Alexander et al., 2020), while electrical stimulation in humans causes hypotension (Lacuey et al., 2018). Although it is possible that variations in acute stressors,

selectivity of cortical subregion targeting, and/or species may account for these divergent findings, all of the described studies used non-specific blockade or stimulation. Given the complexity of cortical circuitry and the numerous local inhibitory mechanisms (McKlveen et al., 2019), cell-type specific targeting has the ability to isolate the precise contribution of vmPFC principal cells independent of afferents, interneurons, and glia. To address this issue, our prior studies developed anatomical and cell-type specific approaches to reduce glutamate outflow from IL pyramidal neurons. Genetic knockdown of IL glutamate release in males increased neuroendocrine and cardiovascular responses to both acute and chronic stress (Myers et al., 2017; Schaeuble et al., 2019), demonstrating the necessity of IL output for inhibiting physiological stress responses. The current cell-type specific optogenetic targeting adds further temporal specificity and identifies the sufficiency of male IL glutamate neurons for stress inhibition. Albeit with opposing effects in females.

The IL does not directly innervate the neurons that govern endocrine and autonomic stress responses. Accordingly, downstream glutamate signaling from IL synaptic terminals requires intermediary synapses. The exact circuits engaged by IL pyramidal cells to bring about the observed effects remain to be determined. However, anterograde mapping studies indicate that IL projections widely innervate the forebrain and brainstem (Vertes, 2004; Wood et al., 2018). We previously found that, in males, stress-activated IL neurons innervate local inhibitory GABAergic neurons in the posterior hypothalamus (PH) (Myers et al., 2016). Furthermore, blocking GABAergic tone in the PH reduces social behavior and increases HPA axis reactivity suggesting inhibition of the PH may be important for limiting behavioral and physiological stress responses (Myers et al., 2016). Additionally, IL inputs to the amygdala are critical for fear extinction and reducing anxiety-like behavior (Sierra-Mercado et al., 2011; Adhikari et al.,

2015). Interestingly, amygdala-projecting IL neurons are both resistant to stress-induced dendritic retraction as well as sensitive to estrogen (Shansky et al., 2009, 2010). Thus, the IL-amygdala circuit could play a key role in sex differences in behavioral regulation. Although the downstream mechanisms of IL sympathetic regulation are unknown, male IL projections target pre-autonomic cell groups in the brainstem and give rise to multi-synaptic pathways that innervate the adrenal medulla (Gabbott et al., 2005; Dum et al., 2019). Further sex-specific analysis of IL synaptic signaling in forebrain and brainstem nuclei is necessary to determine the basis of divergent behavioral and physiological integration.

While ovarian hormones have far-reaching effects on behavior and physiological systems, we did not control for estrous cycle phase. Instead, we used randomly cycling females. Cycle phase was reported for each treatment, but statistical power was insufficient to examine phase as a factor. It remains to be determined how gonadal hormones might contribute to the sexually divergent effects observed. Estrogen receptor (ER) α, β and the g-protein coupled ER are expressed in pyramidal and non-pyramidal PFC neurons in both sexes (Montague et al., 2008; Almey et al., 2014). Further, ER localized to PFC dendritic spines regulates synaptic morphology and ionotropic glutamate receptor ubiquitination/degradation following repeated stress (Hao et al., 2006; Wei et al., 2014; Yuen et al., 2016). These protective effects are dependent on PFC ERα and estradiol-synthesizing aromatase (Wei et al., 2014), indicating a role for extra-ovarian estrogen synthesis. In addition, ER expressed in axons and axonal terminals rapidly alters pre-synaptic transmission in pyramidal cells (Almey et al., 2014). Although studies have described multiple interactions between ER and cortical glutamate signaling (Galvin and Ninan, 2014; Hara et al., 2018), much less is known about PFC progesterone signaling. Progesterone receptors are expressed in frontal cortex (Guerra-Araiza et al., 2003) but

progesterone derivatives also signal through $GABA_A$ receptors and regulate GABA subunit expression (Andrade et al., 2012). Moreover, sex differences in IL regulation of behavior and physiology could arise from the actions of androgens. Androgen receptors (AR) are expressed in PFC neurons and glia (Finley and Kritzer, 1999). AR is also enriched in VTA-projecting PFC neurons that influence extracellular dopamine in PFC through downstream VTA glutamate signaling (Aubele and Kritzer, 2012). It is particularly interesting that androgens and estrogens act in opposition to modify PFC dopamine, norepinephrine, and serotonin metabolism during novel environment stress (Handa et al., 1997). Thus, gonadal steroids and locally synthesized modulators affect PFC cellular processes and projection activity, ultimately engaging multiple neurotransmitter systems. Accordingly, the specific cellular and synaptic processes contributing to sex-dependent stress reactivity and resilience are promising avenues for identifying therapeutic targets.

The current study identified a cortical node integrating mood-related behaviors with endocrine outcomes. Moreover, activity in this vmPFC cell population produced sex-specific effects on behavior and physiology. In addition to highlighting the necessity of sex-based investigation, these data point to a neurochemical basis for sex differences in stress-related health determinants. Ultimately, further investigation of brain-body interactions in the face of prolonged stress may provide a better understating of disease risk and resilience factors.

Funding

This work was supported by NIH grants R00 HL122454 and R01 HL150559 to Brent Myers. Data generated by the University of Cincinnati Mouse Metabolic Phenotyping Core were supported by NIH grant U2C DK059630.

Disclosures

The authors have no conflicts of interest to disclose.

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CHAPTER 3: PREFRONTAL GLUTAMATERGIC PROJECTIONS TO THE POSTERIOR HYPOTHALAMUS REGULATE AFFECTIVE VALENCE AND PHYSIOLOGICAL STRESS REACTIVITY

Preamble

The following results are from an in-prep manuscript. Chapter 2 referred to the prefrontal region of interest as the infralimbic cortex or IL. In this chapter, due to the spread of virus outside the borders of the IL, the region referred to has been expanded to the ventromedial prefrontal cortex, vmPFC. While viral spread necessitates this shift, the effects are still likely predominately from IL neural activity. Reasoning for IL predominance is available in the discussion.

Summary

The ventromedial prefrontal cortex (vmPFC) is heavily associated with major depressive disorder both in humans and preclinical models. Previously, I identified that the activity of glutamatergic vmPFC neurons differentially regulates affective valence, social motivation, and acute stress responding in males and females. However, the nuclei that the vmPFC glutamatergic output signals through to generate these behavioral and physiological changes are undetermined. Here, I utilized an optogenetic approach to stimulate vmPFC glutamatergic projections within the posterior hypothalamus. My results show that vmPFC glutamatergic output to the posterior hypothalamus produces positive valence in males and females yet decreases the glucose stress response in males while increasing the corticosterone response in females.

Background

In Chapter 2, my research established that stimulation of ventromedial prefrontal (vmPFC) glutamatergic neurons has sexually divergent impacts on behaviors associated with mood disorders. In short, in males increasing the activity of vmPFC glutamatergic neurons has a positive valence, increases social motivation, and constrains aspects of the acute endocrine and autonomic stress response. However, in females, there is no valence or social motivational impact associated with vmPFC glutamatergic neural stimulation. Further, contrary to males, stimulation of female glutamatergic vmPFC neurons increases the glucose response to acute stress. To dissect the differential functions of the male and female vmPFC, I utilized similar optogenetic techniques as in chapter 2 to stimulate the activity of vmPFC glutamatergic projections in a possible downstream effector site.

The posterior hypothalamus (PH) regulates social and anxiety-like behaviors as well as acute stress reactivity (Myers et al., 2016). Further, the PH receives dense input from vmPFC glutamatergic neurons (Wood et al., 2018). Pharmacological inhibition of the PH decreases both ACTH and corticosterone response to acute restraint and increases social aggression in males (Myers et al., 2016; Nyhuis et al., 2016). Though the PH is primarily glutamatergic, in males vmPFC pyramidal projections appose a subset of PH GABA neurons, suggesting the vmPFC can regulate the excitatory-inhibitory tone of the PH (Myers et al., 2016). Thus, I sought to test the overall hypothesis that stimulation of IL glutamatergic neurons within the PH would replicate the effects of IL glutamatergic stimulation at the cell bodies, specifically in males it would induce a positive place preference, increase social motivation, and reduce overall physiological stress responding while in females it would not induce a place preference, would not alter social motivation but would augment the sympathetic stress response.

To address this hypothesis, I utilized a similar optogenetic approach as chapter 2 to stimulate glutamatergic vmPFC projections in the PH. Channelrhodopsin-2 (ChR2) was expressed under the calcium/calmodulin-dependent protein kinase type II α (CaMKIIα) and fiber optic cannulas were implanted to enable optogenetic stimulation of vmPFC glutamatergic terminals in the PH (Wood et al., 2018). Like chapter 2, this approach was combined with measures of place preference and social behavior to examine affective valence and sociability. Behavioral assessment was followed by measures of physiological stress reactivity. Ultimately, my findings identify that evoked vmPFC glutamate release in the PH has a positive influence on the affective state in both males and females, does not influence social motivation, and has sexually divergent influences on autonomic and endocrine stress reactivity.

Methods

Animals

Age-matched adult male and female Sprague-Dawley rats were obtained from Envigo (Denver, CO) with male rat weight ranging from 250-300 g and female from 150-200 g. After stereotaxic surgery, rats were housed individually in shoebox cages with cardboard tubes for enrichment in a temperature- and humidity-controlled room with a 12-hour light-dark cycle (lights on at 07:00h, off at 19:00h) and food and water *ad libitum*. Following ARRIVE guidelines, all treatments were randomized, and experimenters blinded. All procedures and protocols were approved by the Colorado State University Institutional Animal Care and Use Committee (protocol: 1321) and complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Signs of poor health and/or weight loss ≥ 20% of pre-surgical weight were *a priori* exclusion criteria.

Microinjections

Rats were anesthetized with isoflurane (1-5%) followed by analgesic (0.6 mg/kg buprenorphine-SR, subcutaneous) and antibiotic (5 mg/kg gentamicin, intramuscular) administration. Rats received bilateral microinjections (1.0 µL) of adeno-associated virus (AAV) into the vmPFC (males: 2.7 mm anterior to bregma, 0.6 mm lateral to midline, and 4.0 mm ventral from dura, females: 2.45 mm anterior to bregma, 0.5 mm lateral to midline, and 4.0 mm ventral from dura). AAV5-packaged constructs (University of North Carolina Vector Core, Chapel Hill, NC) either expressed yellow fluorescent protein (YFP) or ChR2 conjugated to YFP under the synapsin promoter to allow for axon terminal expression in projection neurons (Wood et al., 2018). All microinjections were carried out with a 25-gauge, 2-µL microsyringe (Hamilton, Reno, NV) using a microinjection unit (Kopf, Tujunga, CA) at a rate of 5 minutes/µL. The needle was left in place for 5 minutes before and after injections to reduce tissue damage and allow diffusion. The skin was closed with wound clips that were removed 2 weeks after injections and animals were allowed at least 6 weeks for recovery and ChR2 expression.

Electrophysiology

Adult male rats (n = 5) were injected with AAV constructs as described above and, after 10 weeks, exposed to 5% isoflurane before decapitation and brain removal. As previously described (Rau and Hentges, 2017; Wallace et al., 2021), brains and sections were collected in ice-cold artificial CSF (aCSF) consisting of the following (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2 \Lambda 6H_2O$, 2.4 $CaCl_2 \Lambda 2H_2O$, 1.2 NaH_2PO_4, 11.1 glucose, and 21.4 $NaHCO_3$, bubbled with 95% O_2 and 5% CO_2. Coronal slices containing the PH were cut at a thickness of 240 mm

using a model VT1200S vibratome (Leica Microsystems, Buffalo Grove, VMPFC). After resting 1 hr at 37°C in aCSF, slices were transferred to the recording chamber and perfused with oxygenated 37° C aCSF at a 2 ml/min flow rate. For whole-cell recordings, the internal recording solution contained the following (in mM): KCL 57.5, K-methyl sulfate 57.5, NaCl 20, $MgCl_2$ 1.5, HEPES 5; EGTA 0.1; ATP 2; GTP 0.5, and phosphocreatine 10. The pH was adjusted to 7.3. Recording electrodes had a resistance of 2 – 4 MΩ when filled with this solution. Whole-cell patch-clamp recordings were acquired in voltage-clamp at a holding potential of -60 mV using an Axopatch 200B Amplifier (Molecular Devices, San Jose, CA). Current-clamp recordings were acquired while holding current at 0 pA. Electrophysiological data were collected and analyzed using Axograph X software on a Mac OS X operating system (Apple, Cupertino, CA). Light activation of vmPFC axon terminals expressing ChR2 was triggered via a 473 nm LED (1.1 mW, Thorlabs, Newton, NJ) light pulse driven by a LEDD1B driver (Thorlabs, Newton, NJ) triggered through the TTL output on an ITC-18 computer interface board (HEKA Instruments, Holliston, MA). Voltage-clamp experiments used a 100 ms light pulse while current-clamp experiments used 5 ms pulses. Current-clamp experiments utilized 10 Hz stimulation for 5 min bouts. Recordings were excluded if access resistance exceeded 10 Ω during recording.

Fiber optic cannulae

Rats were anesthetized (isoflurane 1-5%) and bilateral fiber-optic cannulas (flat tip 400/430 μm, NA = 0.66, 1.1 mm pitch with 7.5 mm protrusion for males and 7.0 mm protrusion for females; Doric Lenses, Québec, Canada) were targeted to the PH (males and females: 4.0 mm posterior to bregma, 0.5 mm lateral to midline, and 7.5 mm ventral from dura) and lowered into place. Cannulas were secured to the skull with metal screws (Plastics One) and dental cement

(Stoelting, Wood Dale,). The skin was sutured, and rats were given a subcutaneous injection of analgesic (0.6 mg/kg Buprenorphine-SR) and an intramuscular injection of antibiotic (5 mg/kg gentamicin). Following 1 week of recovery, rats were handled daily and acclimated to the stimulation procedure for another week before experiments began. Rat handling and cannula habituation continued daily throughout experiments.

Optogenetic stimulation

Light pulses (8 mW, 5 ms pulses, 10 Hz) were delivered through a fiber-optic patch cord (240 μm core diameter, NA = 0.63; Doric Lenses) connected to a 473 nm LED driver (Doric Lenses).

Estrous cycle cytology

Following measures of behavior and/or physiology, vaginal cytology was examined to approximate the estrous cycle stage. A damp (deionized water) cotton swab was used to collect cells from the vaginal canal and place them onto a glass slide. Dried slides were viewed under a 10x objective light microscope by a minimum of two blind observers and were categorized as proestrus, estrus, metestrus, or diestrus (Smith et al.; Cora et al., 2015; Solomon et al., 2015).

Real-time place preference

The real-time place preference (RTPP) assay was used to assess the valence of PH-projecting vmPFC terminal neurotransmitter release. Methodology matched the previously published approach (Wallace et al., 2021). Rats were handled to connect LED-connected patch

cords to cannulas for light delivery then placed in a custom-made fiberglass arena with two chambers connected by a corridor for 10 minutes on two consecutive days (chambers: 15 × 15’’, corridor: 8 × 6’’, 15’’ deep). On the first day, no stimulation was delivered on either side. On the second day, rats received 470 nm light throughout the time spent in the assigned stimulation side. Stimulation stopped when rats exited the assigned stimulation side but re-commenced upon re-entry. Thus, rats determined the amount of stimulation received through time spent in the stimulation side. Trials were recorded by a camera mounted above the arena and animal position was tracked by Ethovision software (Noldus Information Technologies) for automated optic hardware control. Stimulation side assignment was counterbalanced, and animal testing was randomized. The time rats spent in the stimulation side was divided by the total time and multiplied by 100 to generate a percentage of time spent in the stimulation side.

Social behavior

A modified version of the 3-chambered social behavior assay was used to accommodate optic patch cords, as previously reported (Moy et al., 2004; Felix-Ortiz and Tye, 2014; Wallace et al., 2021). To examine social interaction, each rat was connected to a patch cord and placed in a black rectangular fiberglass arena (36 x 23’’, 15.8’’ deep). Initially, the arena was empty and experimental rats were allowed to explore for 5 minutes without optic stimulation. The experimental rat was then returned to their home cage while an empty enclosure (ventilated with small round openings) was placed on one side of the arena, defined as the object, and an identical enclosure containing an age- and sex-matched conspecific was placed on the other side of the arena, defined as the social cage. The experimental rat was then placed in the middle of the arena and allowed to explore freely for 10 minutes with 5 ms pulsatile stimulation delivered throughout

to quantify social motivation. The experimental rat was then again placed into its home cage while the empty enclosure was replaced with a new enclosure containing a novel age- and sex-matched conspecific. The experimental rat freely explored for 10 minutes while receiving optic stimulation to assess social novelty preference. Behavior was recorded with an overhead camera and interactions were defined as nose pokes onto cages and scored by a treatment-blinded observer. The duration of interactions was divided by the total time of each interaction period and multiplied by 100 to give a percent interaction value. Sides for object cage, social cage, and the novel cage were counterbalanced and animal order randomized.

Restraint stress

Restraint was used to examine neuroendocrine responses to acute stress. Rats were placed in plastic film decapicones (Braintree Scientific, Braintree, MA) and connected to fiber-optic patch cords for optic stimulation of vmPFC axons throughout the 30 min restraint. Blood samples (approximately 250 µL) were collected by a tail clip at the initiation of restraint with additional samples taken 15 and 30 min after (Vahl et al., 2005). After restraint, patch cords were disconnected, and rats returned to their home cage with recovery blood samples collected at 60 and 90 min after the initiation of restraint. Blood glucose was determined with Contour Next EZ glucometers (Bayer, Parsippany, NJ) and 2 independent readings for each time point were averaged. Blood samples were centrifuged at 3000 X g for 15 min at 4 ◦C and plasma was stored at −20 ◦C until ELISA. Corticosterone is the final product of the hypothalamic-pituitary-adrenal (HPA). Plasma corticosterone, as the final product of HPA axis, was measured using an ENZO Corticosterone ELISA (ENZO Life Sciences, Farmingdale, NY) with an intra-assay coefficient of variation of 8.4% and an inter-assay coefficient of variation of 8.2% (Bekhbat et al., 2018;

Dearing et al., 2021). All samples were run in duplicate, and all time points were run in the same assay.

Data analysis

Data are expressed as mean ± standard error of the mean. Data were analyzed using Prism 8 (GraphPad, San Diego, CA), with statistical significance set at $p < 0.05$ for rejection of null hypotheses. RTPP stimulation preference was assessed via repeated measure two-way analysis of variance (ANOVA) with virus and day (repeated) as factors. In the case of significant main or interaction effects, Sidak's multiple comparisons were run to determine group differences. Social motivation and novelty preference were assessed with unpaired t-tests to compare virus groups within social, object, novel rat, or familiar rat interactions. Total distance traveled during RTPP, and social interaction was assessed with unpaired t-tests comparing virus groups. Stress responses over time (corticosterone, glucose) were analyzed using mixed-effects analysis with virus and time (repeated) as factors, followed by Fisher's post hoc if significant main or interaction effects were present.

Results

Validation and design

AAV viral vectors were targeted to the vmPFC for expression of membrane-targeted ChR2-YFP or cytosolic YFP under the pyramidal neuron promoter, CaMKIIα (**Figure. 5A**). Fiber optic cannulas implanted in the PH permitted stimulation of vmPFC glutamatergic axons within the PH. Whole-cell patch-clamp recordings in slice demonstrated 10 Hz stimulation of vmPFC axons led to high-fidelity action potential generation in PH neurons (**Figure. 5B**). Males

and females underwent the same experiment design, (**Figure. 5C**) further detailed in the methods. Male and female cohorts were run separately. After experimental conclusion, the placement of viral injections and fiber optics was determined. Only animals with injections primarily targeted to the vmPFC (**Figure. 5D**), as well as with cannula placement verified to be within or immediately (within 0.5 mm) dorsal to the PH were included in analyses (**Figure. 5E**).

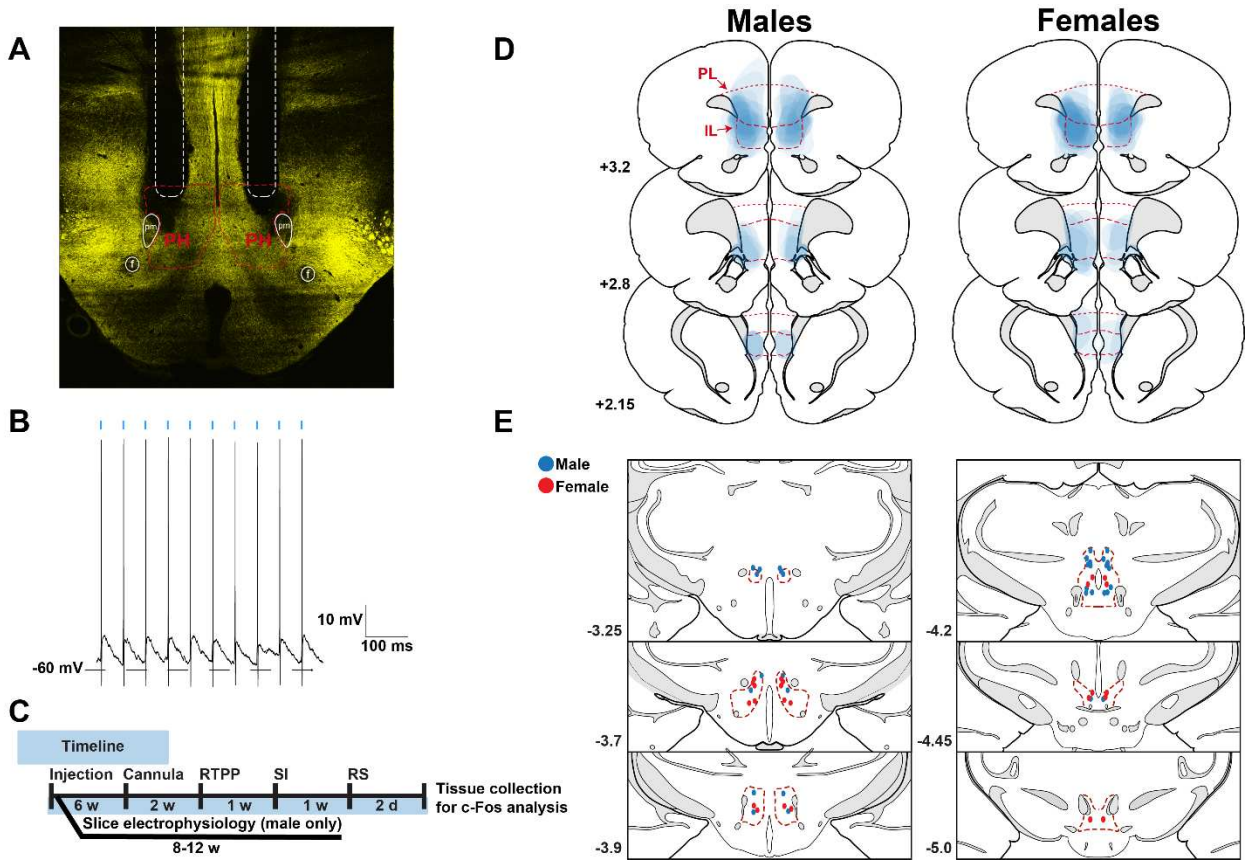

Figure 5: Approach validation and experimental design. (**A**) Photomicrograph of vmPFC ChR2-YFP in the hypothalamus. Injection of AAV-packaged constructs led to the expression of cytosolic YFP or membrane-targeted ChR2-YFP under the CaMKIIα promoter. (**B**) Current-clamp recordings found stimulation-locked spiking with 10 Hz stimulation (1.1 mW, 5 ms pulse). (**C**) Experimental timelines. RTPP: real-time place preference, SI: social interaction, RS: restraint stress (**D**) AAV injection spread was mapped within the vmPFC, blue. (E) Optic fiber placements (Male: blue, Female: red) were mapped within the PH (red outline). Coronal sections adapted from Swanson Rat Brain Atlas (3rd edition).

Real-time place preference

RTPP was used to examine the affective valence of stimulating vmPFC terminal release within the PH, similar to previous work (Wallace et al., 2021). Cumulative heat maps illustrate time spent in the stimulation versus non-stimulation chambers (**Figure. 6A**). Both male and female rats showed a preference for the stimulation chamber (**Figure. 6B**, Males repeated-measures 2-way ANOVA: stimulation $F(1, 29) = 6.312$, $p < 0.05$, ChR2 $F(1,29) = 12.66$, $p < 0.01$, stimulation x ChR2 $F(1, 29) = 23.94$, $p < 0.0001$, Females repeated-measures 2-way ANOVA: stimulation $F(1, 22) = 17.11$, $p < 0.001$, ChR2 $F(1,22) = 0.3014$, $p > 0.05$, stimulation x ChR2 $F(1,22) = 3.566$, $p > 0.05$). Specifically, Sidak's post hoc indicated a preference for the stimulation chambers compared to habituation day in the ChR2 groups of males and females (Males: $n = 16$, $p < 0.0001$, Females: $n = 12$, $p < 0.001$), though in across-group comparisons only males had a ChR2 vs.YFP effect on stimulation day ($n = 15$-16, $p < 0.0001$). vmPFC axonal stimulation did not affect general locomotor activity in either sex (**Figure. 6C**).

Social behavior

The three-chamber social interaction assay was used to determine the influence of PH-targeted vmPFC glutamatergic output on sociability. There was no difference in social interactions with stimulation in either sex (**Figure 6D;** Males: $n = 15$/group, unpaired t-test: ChR2 vs YFP $t(28) = 0.099$, $p = 0.92$, Females: $n = 11$/group, unpaired t-test: ChR2 vs YFP $t(20) = 0.35$, $p = 0.73$). Further, vmPFC-PH axonal stimulation did not affect preference for novel vs. familiar interactors (Data not shown). Overall, these results indicate that vmPFC glutamatergic release within the PH does not alter social behavior.

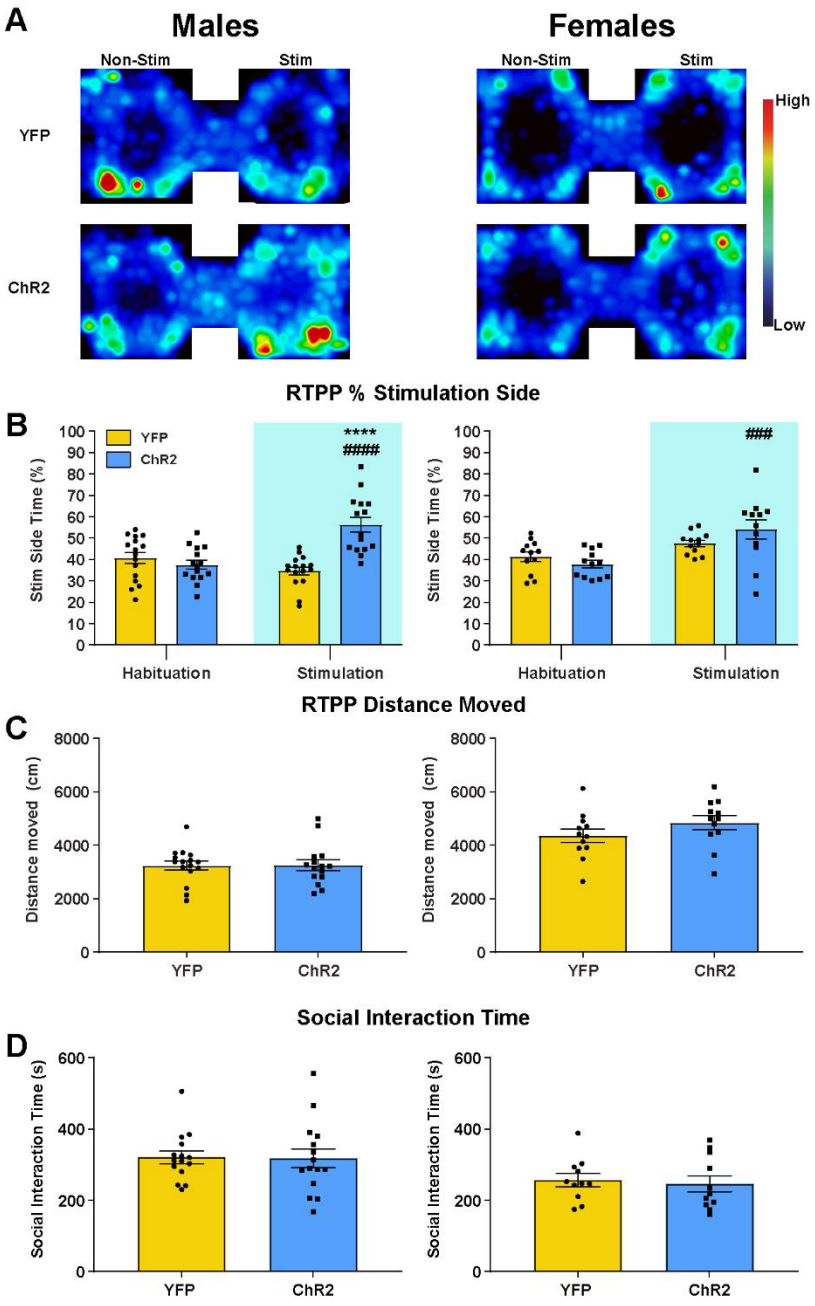

Figure 6: Stimulation of PH-targeted vmPFC terminals has a positive valence in both sexes but does not alter social behavior. (**A**) Heat maps illustrate mean animal position in the RTPP arena on stimulation day (n = 10-15/group). (**B**) Male ChR2 rats preferred the chamber paired with stimulation (blue shading), relative to habituation day and YFP controls. Female ChR2 rats preferred the stimulation chamber relative to habituation day. **** $p < 0.001$ vs. YFP within stimulation. #### $p < 0.0001$ vs. habituation within ChR2. (**C**) Total distance traveled during stimulation found no treatment-based differences in locomotion. (**D**) Social Interaction time, defined as nose-poke onto the social cage, was not altered by ChR2 stimulation in males or females.

Endocrine reactivity

To determine the effect of stimulating vmPFC axons within the PH on autonomic and HPA responses, blood glucose and plasma corticosterone were monitored during restraint stress respectively. In males, optic stimulation did not alter corticosterone (n = 16-17/group, mixed-effects: time $F_{(4,114)}$ =74.15, p < 0.0001, ChR2 $F_{(1,31)}$ = 0.4775, p = 0.49). However, stimulation did decrease blood glucose (mixed-effects: time $F_{(4,119)}$ = 34.59, p < 0.0001, ChR2 $F_{(1,31)}$ =1.349, p = 0.25, time x ChR2 $F_{(4,119)}$ = 1.161, p = 0.3315) in male rats during restraint (**Figure. 7B**; 15 min, p < 0.01). In contrast, stimulation increased corticosterone in female rats during the recovery period (**Figure. 7C**; n = 12-13/group, mixed-effects: time $F_{(4,99)}$ = 23.91, p < 0.0001, ChR2 $F_{(1,99)}$ = 3.491, p = 0.065, time x ChR2 $F_{(4,99)}$ = 2.323, p = 0.062), yet did not alter glucose response. Collectively, these results suggest that PH-targeted vmPFC glutamatergic release in males reduces sympathetic activity but does alter HPA axis. In females, PH targeted vmPFC glutamatergic release increases HPA axis activity but does not alter autonomic activity.

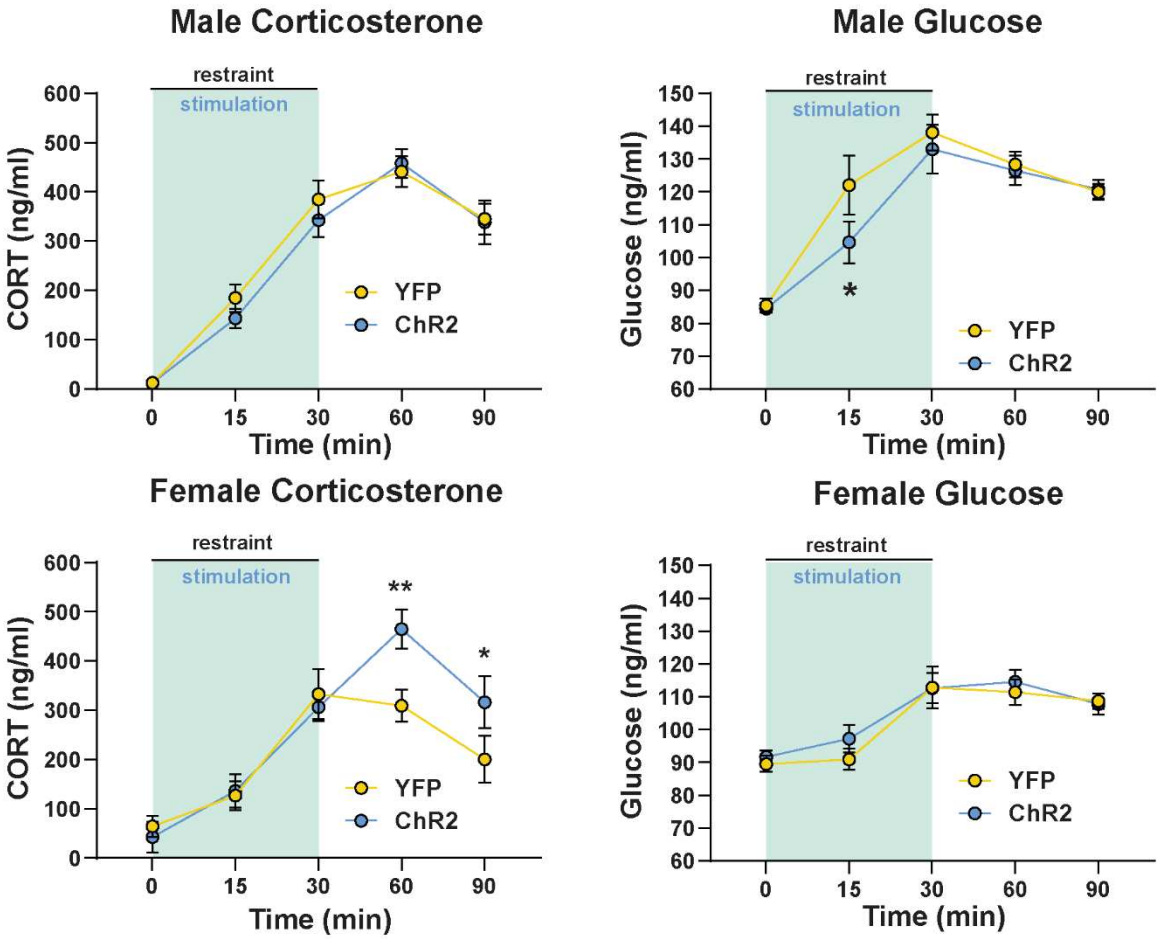

Figure 7: Stimulation of PH-targeted vmPFC projections reduces male glucose response but increases female corticosterone response to novel restraint stress. (A) Stimulation (blue shading) during restraint did not alter corticosterone but did reduce blood glucose **(B)** in ChR2 males. **(C)** Stimulation in females increased corticosterone responses following termination of stress but did not alter glucose **(D)**. n = 10-17/group, * $p < 0.05$, ** $p < 0.01$ vs. YFP.

Discussion

In this study, I utilized optogenetic stimulation to determine how vmPFC glutamatergic output to the PH alters behavior and acute stress reactivity. My results show that vmPFC glutamatergic output to the PH has a positive affective influence in both sexes but does not alter social behaviors. Further, vmPFC glutamatergic output in the PH reduces the sympathetic stress response in males but increases HPA axis response in females.

Due to the viral spread outside the boundaries of the IL, the circuit being tested is referred to as vmPFC to PH. However, the observed phenotypes are likely mediated primarily through IL to PH projections due to both the substantially higher viral YFP expression in the IL compared to the prelimbic cortex (PL), and the more abundant projections to the PH from the IL compared to the PL. Further, neurons projecting from the IL to the PH show substantially higher stress-activation than PL to PH-projecting neurons, suggesting the IL to PH circuits plays a more significant role in acute stress responding (Myers et al., 2016). Thus, while PL to PH circuits influence the results of my stimulation, they are predominantly IL to PH circuits.

Further, the possibility that the phenotypes arising from my stimulation of IL projections to the PH might be due to off-target effects in other regions cannot be entirely discounted. However, efferent tracing studies of the IL show that the projections to the PH are considerably greater in magnitude than to surrounding regions such as the dorsomedial and lateral hypothalamus (Wood et al., 2018). Yet, excluding the possibility that the stimulation might act through other hypothalamic or brainstem nuclei such as the periaqueductal gray will require more work employing specific targeting of these projection pathways.

In chapter 2, I established that IL glutamatergic output has sexually divergent impacts on behavior and acute stress reactivity. This study improves upon that work by determining how specific vmPFC projections to the PH contribute to the regulation of behavior and stress reactivity. PH-specific vmPFC glutamatergic output in males recreates the positive affect that I found with IL somatic stimulation. Further PH-specific vmPFC glutamatergic output also mimicked the constraint of the male glucose stress response, however, it did not alter the corticosterone response. Intriguingly, PH-specific glutamatergic output has a positive affective influence in females and increases the corticosterone stress response, neither of which was

identified with IL somatic stimulation. This difference may be accounted for by other vmPFC output targets that oppose the vmPFC to PH circuit influence. The vmPFC has wide-ranging projections throughout limbic and autonomic-associated regions. Of particular note, the vmPFC projects to the basolateral amygdala, which has previously been associated with regulating social behavior in females (Huang et al., 2020). Further, the vmPFC projects widely throughout hypothalamic nuclei including the dorsomedial and lateral hypothalamus as well as to the GABAergic periphery of the PVN (Vertes, 2004; Wood et al., 2018). The vmPFC also projects directly to autonomic-regulatory brainstem nuclei, including the nucleus of the solitary tract and monoaminergic nuclei (Hurley et al., 1991; Vertes, 2004; Gabbott et al., 2005).

The observed sex differences in autonomic and HPA axis stress responding could arise from vmPFC projections targeting different neural subtypes within the PH. Male IL projections appose GABAergic neurons in the PH (Myers et al., 2016), but possible sex differences have not been investigated. Differences could also arise from differential PH projections between males and females. PH projections have only been traced in males where they appose neurons expressing corticotrophin-releasing hormone and a variety of other nuclei, including reciprocal connectivity into the vmPFC (Ulrich-Lai et al., 2011; Myers et al., 2016). To my knowledge, there are no female-focused PH projection tracing studies, making comparisons of PH influence difficult. Thus, the underlying mechanisms of the observed sex-differences could be explained by differential action of vmPFC influence on PH excitatory/inhibitory tone, and/or possible differences in PH projections.

Overall, the current study identified a downstream region through which vmPFC glutamatergic neurons regulate behavior and physiological functions. Further, this vmPFC-PH circuit regulates behavior similarly across sex, but differentially regulates stress reactivity. These

findings highlight a circuit that could explain differential health outcomes in males and females, while highlighting the need to further dissect specific vmPFC circuits regulating mood disorder-disrupted functions.

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CHAPTER 4: CHRONIC STRESS IMPACTS ON INFRALIMBIC GLUTAMATERGIC NEURAL ACTIVITY ARE DEPENDENT UPON CONTEXT, SEX, AND OVARIAN HORMONES

Preamble

The following results are from an in-prep manuscript.

Summary

In chapter 2, I utilized optogenetics to increase the activity of infralimbic glutamatergic neurons regardless of their projection targets. I identified that this stimulation produced a positive valence, increased social motivation, and constrained both the HPA and sympathetic response to stress in males. In females, it did not produce an affective valence, did not alter social motivation, and increased the female glucose response to stress. In chapter 3, I identified that stimulation of ventromedial prefrontal axons within the posterior hypothalamus produced positive valence in males and females, did not alter social motivation, and constrained the sympathetic stress response in males while augmenting the HPA response in females. However, while the optogenetic stimulation work has identified behavioral and physiological properties ventromedial prefrontal glutamatergic neurons can regulate, the real-time activity of these neurons during behavior remained unknown. Here, I utilized a fluorescent calcium indicator to record the activity of infralimbic glutamatergic neurons during behavior and stress responding in males, females, and ovariectomized females with and without a history of chronic stress. My results show that IL glutamatergic neurons are responsive to social interactions in all groups, and chronic stress reduces this activity in males and ovariectomized females. Further IL

glutamatergic neurons increase in activity to a novel stressor only in stress-naïve males, however, both stress-exposed and stress-naïve females showed increased neural activity to the stressor. Finally, IL glutamatergic neurons increase in activity to food reward acquisition in all groups, and this activity is altered by chronic stress in males.

Background

Major depressive disorder (MDD), characterized by reduced motivation, anhedonia, and social isolation is the leading cause of years lived with disability worldwide (Friedrich, 2017). Yet, despite the prevalence of MDD, little is known regarding the neurobiological mechanisms that underly its onset. Significant sex differences in prevalence further compound the difficulty, with females at nearly twice the risk of MDD (Kuehner, 2017). Epidemiological evidence implicates life stressors as a primary risk factor for the onset of mood disorders (Grippo and Johnson, 2009; Binder and Nemeroff, 2010; Myers et al., 2014; Sgoifo et al., 2015). Understanding the mechanisms of MDD symptoms requires determining the activity patterns of depression-associated neural populations in association with behavioral states, i.e., the neural encoding of behavior, and how this encoding is disrupted by stress exposure.

The ventromedial portion of the prefrontal cortex (vmPFC) participates in numerous cognitive and emotional processes and is strongly associated with MDD (Krishnan and Nestler, 2008; Wallace and Myers, 2021; Wood and Grafman, 2003; Myers-Schulz and Koenigs, 2012; McKlveen et al., 2015). In particular, Brodmann's Area 25 (BA25), a subregion of the vmPFC, is activated by sadness-provoking stimuli, responds to social isolation, and has reduced volume in MDD patients (Liotti et al., 2000; Beckmann et al., 2009; Vijayakumar et al., 2017). Yet, attempts to elucidate the functional changes in BA25 during pathology or recovery have yielded

mixed results. Mixed-sex studies have reported both reduced metabolic activity in MDD patients (Drevets et al., 1997), and hyperactivity in treatment-resistant depression measured by cerebral blood flow (Mayberg et al., 2005). Thus, while functional vmPFC changes contribute to MDD, how specific vmPFC neural populations are altered in pathology remains unknown.

The infralimbic cortex (IL) is the rodent anatomical homolog of the human BA25. The IL contains glutamatergic projection neurons and local network inhibitory neurons (McKlveen et al., 2015, 2016; Wood et al., 2018). IL glutamatergic neurons project widely throughout the limbic systems, providing targets for the regulation of behavioral and endocrine processes (Vertes, 2004; Wood et al., 2018). Further, IL neural populations can regulate social and motivational states, as well as acute stress reactivity and glutamatergic output from the IL is necessary for behavioral consequences following chronic stress exposure (Pace et al., 2020a; Wallace and Myers, 2021). Yet, while chronic stress consistently induces dendritic hypertrophy in IL glutamatergic neurons (Cerqueira et al., 2005; Goldwater et al., 2009; Shansky et al., 2009; Luczynski et al., 2015; Czéh et al., 2018), the functional consequences of chronic stress exposure have been mixed with some studies providing evidence of IL hyper-inhibition (Gilabert-Juan et al.; McKlveen et al., 2016; Shepard et al., 2016), and others hypo-inhibition (Czéh et al., 2018; Ghosal et al., 2020). Further, sexual divergence has been identified in chronic-stress-induced changes in IL neural characteristics, such as dendritic spine density, an effect modulated by ovarian hormones (Shansky et al., 2010; Wei et al., 2014; Anderson et al., 2019). Thus, while research has established that stress alters attributes of IL neurons, how IL glutamatergic neurons encode behavioral states in real-time, how this encoding is perturbed by chronic stress exposure, and the role of sex and ovarian hormones remains unknown.

To identify how specific vmPFC neural populations encode behaviors, I recorded the activity of genetically identified IL glutamatergic projection neurons with a fluorescent calcium indicator. Specifically, to permit the recording of IL glutamatergic neurons in rats during behavioral testing, GCaMP6s was expressed under the calcium/calmodulin-dependent protein kinase type II α (CaMKIIα) promoter followed by the implantation of an IL targeted fiber-optic cannula to excite and collect GCaMP6s fluorescence. Rats either remained stress-naïve or were exposed to two weeks of chronic variable stress (CVS) followed by behavioral testing. Behavioral tests included interaction with an object and social stimulus, exposure to restraint stress, and hedonic food reward. Based on previous work indicating a role for IL glutamatergic neurons in regulating object and social interactions, reward acquisition, and stress responding, I sought to test the hypothesis that IL glutamatergic neurons would have increased activity during these behaviors in males and females. Further, chronic stress exposure would reduce IL glutamatergic neural response to stimuli in males with minimal alterations to female activity. Finally, an ovariectomized (OVX) cohort was run to test the hypothesis that females without protective ovarian hormone actions would have reduced IL glutamatergic neural activity following chronic stress similar to males.

Methods

Animals

Age-matched adult male, female, and ovariectomized (OVX) female Sprague-Dawley rats were obtained from Envigo (Denver, CO) with male rat weight ranging from 250-300 g, female from 150-200 g, OVX females 250-300 g. Ovariectomy occurred two weeks before arrival. After stereotaxic surgery, rats were housed individually in shoebox cages with cardboard

tubes for enrichment in a temperature- and humidity-controlled room with a 12-hour light-dark cycle (lights on at 07:00h, off at 19:00h) and food and water *ad libitum*. Per ARRIVE guidelines, all treatments were randomized, and experimenters were blinded. All procedures and protocols were approved by the Colorado State University Institutional Animal Care and Use Committee (protocol: 2129) and complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Signs of poor health and/or weight loss ≥ 20% of pre-surgical weight were a priori exclusion criteria.

Microinjection and Cannulation

Microinjections and cannulations were performed as previously described (Wallace et al., 2021). Rats were anesthetized with isoflurane (1-5%) and administered analgesic (0.6 mg/kg buprenorphine-SR, subcutaneous). Rats received unilateral microinjections (Males 1.5 µL, Females 1 µL) of adeno-associated virus (AAV) into the IL (males: 2.7 mm anterior to bregma, 0.6 mm lateral to midline, and 4.2 mm ventral from dura, females: 2.3 mm anterior to bregma, 0.5 mm lateral to midline, and 4 mm ventral from dura). AAV9-packaged constructs (Addgene, virus # 107790) expressing GCaMP6s under the CaMKIIα promoter to achieve pyramidal cell-predominant expression (Wood et al., 2018). The needle was left in place for 5 minutes before and after injections to reduce tissue damage and allow diffusion. All microinjections were carried out with a 25-gauge, 2-µL microsyringe (Hamilton, Reno, NV) using a microinjection unit (Kopf, Tujunga, CA) at a rate of 5 minutes/µL. Following injection, unilateral fiber-optic cannulas (flat tip 400/430 µm, NA = 0.57, 5 mm protrusion; Doric Lenses, Québec, Canada) were aligned with the IL injection sites and lowered to the IL. Cannulas were secured to the skull with metal screws (Plastics One) and dental cement (Stoelting, Wood Dale, IL). Skin was

sutured and, following 1 week of recovery, rats were handled daily and acclimated to the recording procedure for another week before experiments began. Rat handling and cannula habituation continued daily throughout experiments.

Chronic Variable Stress

As previously performed (Wallace et al., 2021), chronic variable stress was comprised of twice daily (AM and PM) repeated and unpredictable stressors presented in a randomized manner including: exposure to a brightly-lit open field (28.5 x 18'' 13'' deep, 1 hour), cold room (4° C, 1 hour), cage tilt (45°, 1 hour), forced swim (23° to 27° C, 10 minutes), predator odor (fox or coyote urine, 1 hour), restraint (1 hour), and shaker stress (100 rpm, 1 hour). Additionally, overnight stressors were variably included, comprised of damp bedding (400 mL water) and overnight light. During the 2 weeks of CVS, rats were weighed every 3-4 days to monitor body weight.

Food-restriction

To assess the influence of caloric deficit on IL glutamatergic neural response to food reward, a separate cohort of male rats underwent food-restriction to approximately match CVS-induced reduced food intake (Flak et al., 2011; Schaeuble et al., 2019). Following two weeks of recovery after injection and cannulation, male rats received 16 grams of standard chow at lights off and 2 grams of standard chow at lights on to prevent fasting. Rats underwent food-restriction for 14 days with food reward testing performed on day 15. Like all other cohorts, food-restricted rats were given chocolate chips for two days before test day.

Estrous cycle cytology

All female rats were housed in the same room and randomly cycling. Immediately following acute measures of behavior and physiology, vaginal cytology was examined to approximate the estrous cycle stage as previously described (Wallace et al., 2021). A damp (deionized water) cotton swab was used to collect cells from the vaginal canal and place them onto a glass slide. When dried, slides were viewed under a 10x objective light microscope by a minimum of two blind observers and were categorized as proestrus, estrus, metestrus, or diestrus (Smith et al.; Cora et al., 2015; Solomon et al., 2015).

Fiber Photometry

To excite and record emitted GCaMP6s fluorescence, fiber optic cannula were connected to a Doric fiber photometry system (Doric: 1-site 2-color Fiber Photometry System). Excitation was achieved through a 465 nm wavelength LED modulated at 512 Hz. Movement control was achieved with 405 nm wavelength LED modulated at 208 Hz. Power delivery was approximately 3 mW at cannula tip, measured with a photodiode sensor (PM160, Thorlabs Inc, Newton, NJ). To allow for rodent movement, rats were connected to the Doric system through a pigtailed rotary joint patch cord. Emitted fluorescence was captured with a photon detector (Newport Model: 2151). To separate the components of the 465 nm and 405 nm excitation a lock-in amplifier filtered the incoming fluorescence into the 512 Hz and 208 Hz components respectively with a 12 Hz band filter. Before data collection autofluorescence was reduced by running the 465 and 405 nm LEDs at full power for 30 minutes prior to testing. Further, the optical interface between the implanted rat fiber optic and the patch cord was cleaned with 10%

ethanol and dried immediately before testing. Photometry recording data was acquired at 1200 Hz and down sampled to 30 Hz for analysis (MATLAB).

Before analysis of fiber photometry data, 465 nm and 405 nm components of the excited 512 nm collected fluorescence were analyzed by a treatment-blinded observer for movement artifacts. If movement artifacts were detected the recorded trials were excluded from the analysis. Photometry signal analysis and z-score calculation was performed using MATLAB (version 2019b, MathWorks).

Behavioral Recording

Trials were recorded by a camera mounted above the arena (Noldus Information Technologies) for automated optic hardware control. A transistor-transistor logic pulse at the beginning of video acquisition was used to ensure timeclock with photometry recordings.

Modified Object and Social Assay

A modified version of the 3-chambered social behavior assay was used to accommodate optic patch cords (Felix-Ortiz and Tye, 2014; Moy et al., 2004). To examine social interaction, each cannula was connected to the recording patch cord and the rat was placed in a black rectangular fiberglass arena (36 x 23'', 15.8'' deep). To reduce arena novelty, rats were habituated to the interaction arena for 3 days, 10 minutes a day prior to testing. Further, to reduce stress response and novelty, interactor rats were habituated to being placed within cages for 3 days, 20 minutes a day prior to testing. On testing day, the 14th and final day of CVS, rats were connected to fiber optic patch cords and placed in the center of the arena while photometry

recording was collected. Initially, the arena was empty and experimental rats were allowed to explore for 5 minutes. At 5 minutes, an empty cage, novel to the animals, was placed in the center of the arena. Rats were allowed to freely explore and interact with the empty cage for 5 minutes, after which the empty cage was removed. The arena remained empty for 2 minutes with the experimental rat still freely exploring. After 2 minutes since the removal of the empty cage, a new cage with a novel conspecific rat inside was placed in the center of the arena for 5 minutes. After 5 minutes, the social cage was removed, and experimental rats remained in the arena for 3 more minutes. Interactions were hand scored by a treatment-blinded observer as nose pokes onto the empty cage, object interactions, or cage containing a conspecific, social interaction. Ovariectomized experimental rats interacted with similarly ovariectomized females.

Photometry analysis was performed by time-locking video acquisition with photometry signal as described above. The photometry signal was then aligned with the video start. 465 nm component signal was median Z-scored with a rolling 5-minute median and median absolute deviation (MAD), then aligned to interactions and averaged within an animal. The within-animal averaged signals were then averaged within groups to perform statistical analysis. To determine if GCaMP6s signal was higher during object, social or chocolate interactions, 7 second intervals not containing interactions were randomly selected and the average GCaMP6s signal was measured during the middle 2 seconds of these periods. To analyze signals during interaction periods, social behaviors were filtered to include interactions that were 2 seconds in length or greater and preceded by a 3 second period of non-interaction. Due to fewer instances of object interactions, object interactions were filtered to include interactions that were 1 second in the length or greater and preceded by 3 seconds of non-interaction. The first social and object interactions were analyzed separately to examine the novelty component. To analyze the signal

leading up to interaction, social interactions were filtered to only include interactions with a 10-second or longer period of non-interaction.

Restraint stress

To examine the neural response to stress animals underwent restraint. Rats were connected to the photometry system while in their home cage for 5 minutes to capture a baseline of neural activity. Rats were then unhooked from the photometry systems, restrained in plastic film decapicones, and tethered to the photometry system for 30-minutes while connected to fiber-optic patch cords to capture GCaMP6s fluorescence. To assess endocrine reactivity blood samples (approximately 250 uL) were collected by tail clip at the end of restraint (Vahl et al., 2005). After restraint, rats were returned to their home cage. Blood glucose was determined with Contour Next EZ glucometers (Bayer, Parsippany, NJ) and 2 independent readings for each time point were averaged. Blood samples were centrifuged at 3000 X g for 15 minutes at 4° C and plasma was stored at –20° C until ELISA. Plasma corticosterone was measured using an ENZO Corticosterone ELISA (ENZO Life Sciences, Farmingdale, NY) with an intra-assay coefficient of variation of 8.4% and an inter-assay coefficient of variation of 8.2% (Bekhbat et al., 2018; Dearing et al., 2021). All samples were run in duplicate and all time points were run in the same assay.

The 465 nm photometry signal was median Z-scored with a rolling 1-minute window within 5-minute bins to match the home cage period. Peaks were then detected as values exceeding 2.91 MADs above the median for every 5 minutes (Gunaydin et al., 2014; Muir et al., 2018). The transient frequency for the 5-minute bins was averaged to generate the average transient frequency during restraint. To constrain detection to absolute peaks, parameters were

set for a minimum of 0.3 seconds between peaks and prominence of 1 MAD above the surrounding signal (MATLAB).

Chocolate

To examine IL glutamatergic neural response to freely grabbing a food reward, animals were acclimated to eating chocolate chips. A chocolate chip was placed in each animal's cage at lights off following the final restraint, and then for the next two days at light on and light off. Further, animals were acclimated to the testing environment, home cage in a behavioral testing room, for 15 minutes a day for 2 days. On testing day, animals were handled, and their cannulas were connected to the fiber photometry recording system. Animals were then placed in their home cage without the topper and allowed to freely behave for 3 minutes prior to the chocolate chip being added to the cage. At approximately 3 minutes, a chocolate chip was placed in a corner of the home cage. If animals took longer than 30 minutes to consume the chocolate chip they were excluded from the analysis.

Study Design

An example of the study design is pictured in Figure 1. Due to the normalization required for photometry analysis, data from male, female, and OVX female studies is shown in the same figures. However, data was collected across 3 experiments. Experiment 1 was run with stress-naïve and CVS exposed males (n = 15/group). Following the identification of CVS changes to male IL glutamatergic neural activity during food reward acquisition, a group of food-restricted males was added (n = 7). Experiment 2 was run in stress-naïve and CVS-exposed females (n=

10/group). Finally, following different CVS effects on male and female IL glutamatergic neural activity, an experiment of OVX females was run to assess the effects of ovarian hormone loss (n= 11/group).

Data analysis

Data are expressed as mean ± standard error of the mean. Data were analyzed using Prism 8 (GraphPad, San Diego, CA), with statistical significance set at $p < 0.05$ for rejection of null hypotheses. Weights by treatment and day were analyzed using repeated-measure two-way analysis of variance (ANOVA), followed by Fisher's post hoc if significant main or interaction effects were present. Object, social, and reward interactions were analyzed via unpaired t-tests between stress conditions or random vs interaction comparisons. Peak Z-Score in the seconds preceding a social interaction, as well as glucose and corticosterone values across restraint days were analyzed using mixed-effects analysis with stress and time (repeated) as factors, followed by Fisher's post hoc if significant main or interaction effects were present. Transient Hz were analyzed using mixed-effects analysis with restraint (repeated) and CVS as factors, followed by Sidak's post hoc if significant main or interaction effects were present. Male body weight change was analyzed by one-way ANOVA, followed by Tukey's post hoc if significant main or interaction effects were present.

Results

Validation and design

AAV viral injections were targeted to the IL for expression of GCaMP6s under the regulation of CaMKIIα to target glutamatergic neurons (**Figure. 8A**). Fiber optic cannulas were

targeted to the IL to allow excitation and collection of GCaMP6s fluorescence (Gunaydin et al., 2014; Muir et al., 2018). All cohorts underwent the same experimental design (**Figure. 8B),** OVX female rats underwent ovariectomy surgery 2 weeks before the start of the experiment. Fiber optic placement was determined after the experimental conclusion. Only animals with cannula placement verified to be within or immediately (within 0.2 mm) dorsal to the IL were included in analyses (**Figure. 8C**). Animals were weighed before the beginning of treatment, 2-weeks of CVS or No CVS, and the end of the treatment period. In all groups post-treatment weight was higher in the No CVS group compared to day 1 measurements (Males: repeated-measures 2-way ANOVA: Time x Stress $F_{(1, 19)} = 10.66$, $p < 0.01$, Females: repeated-measures 2-way ANOVA: Time x Stress $F_{(1, 18)} = 8.15$, $p < 0.05$, OVX Females: repeated-measures 2-way ANOVA: Time x Stress $F_{(1, 20)} = 209.2$, $p < 0.0001$). In males and OVX females, CVS exposed animals had lower weight than No CVS on day 13 (**Figure. 8D**). Consistent with previous OVX studies, OVX females had higher weight prior to experiment than cycling females (n = 20-22/group, unpaired t-tests: Female vs OVX Female weight $t_{(40)} = 18.61$, $p < 0.0001$, Chen and Heiman, 2001; Fang et al., 2015).

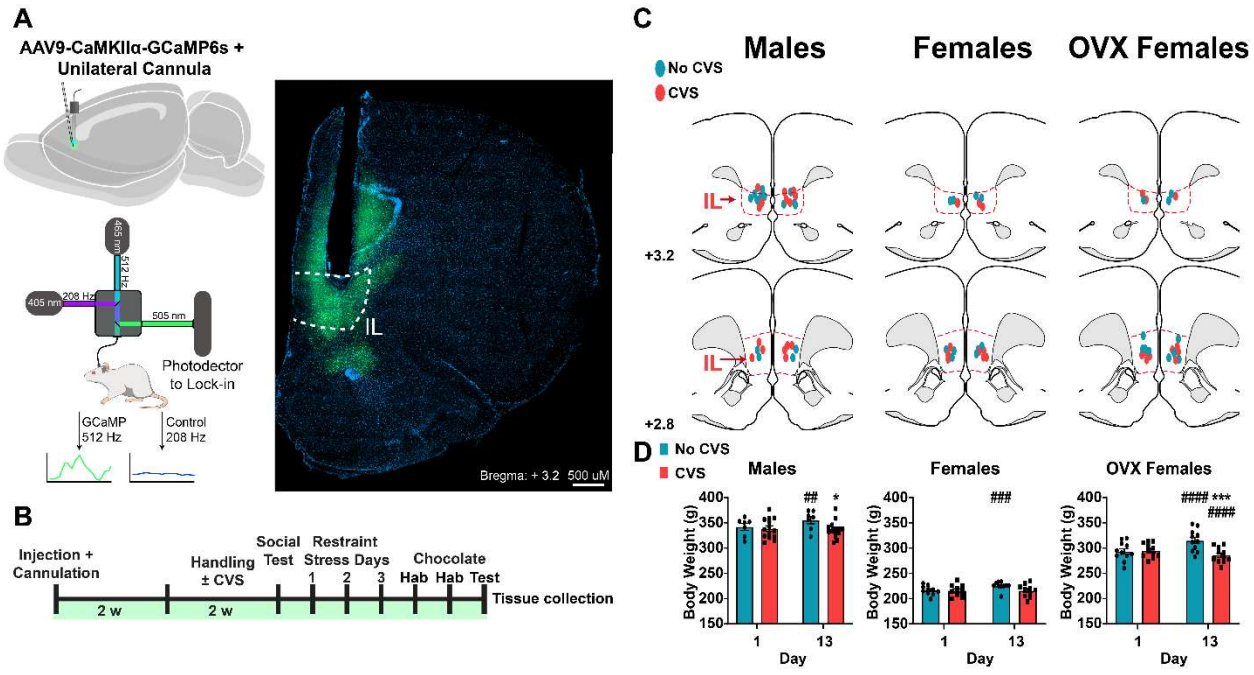

Figure 8: Experimental Design. (**A**) Top left: Schematic of AAV injections and cannulation, Bottom left: schematic of fiber photometry recording system, Right: photomicrograph of GCaMP6s expression in the IL (**B**) Experimental timeline CVS: Chronic Variable Stress. (**C**) Mapped fiber optic cannula positions within or around the IL (red outline) (**D**) Body weight of animals prior to stress condition, CVS or no CVS. Comparisons within-treatment between timepoints: ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$. Comparisons between treatments: * $p < 0.05$, *** $p < 0.001$. OVX: ovariectomized.

Object Interactions

To determine how IL glutamatergic neurons encode interactions with a novel object, experimental rats were allowed to freely interact with an object for 5 minutes. Object interactions were scored as nose pokes onto the empty cage (**Figure. 9A**). Photometry signal was averaged within animals then within groups during object interactions (**Figure. 9B**). During the first object interaction, CVS males had a higher mean signal than stress-naïve males (n = 8-10/group, unpaired t-tests: No CVS vs CVS $t_{(16)} = 2.483$, $p < 0.05$, **Figure. 9C**). Stress-naïve males, females, and OVX females all had higher mean signal during object interactions than random intervals (Random vs Object unpaired t-tests: Males: n = 8 $t_{(14)} = 4.75$, $p < 0.001$, Females: n =7

$t_{(12)} = 5.8$, $p < 0.0001$, OVX n =5 $t_{(8)} = 3.1$ $p < 0.05$, **Figure. 9E**). When comparing subsequent object interactions there was no effect of CVS in any group (data not shown). Further, the mean signal during the first half-second of object interactions was not different in males and OVX females but trended towards significance in intact females (Females unpaired t-test: n = 7-8/group $t_{(13)} = 2.12$ $p = 0.054$ **Figure 9F**). Overall, IL glutamatergic neurons increase in activity during object interactions, and CVS increases male responses to first the novel interaction.

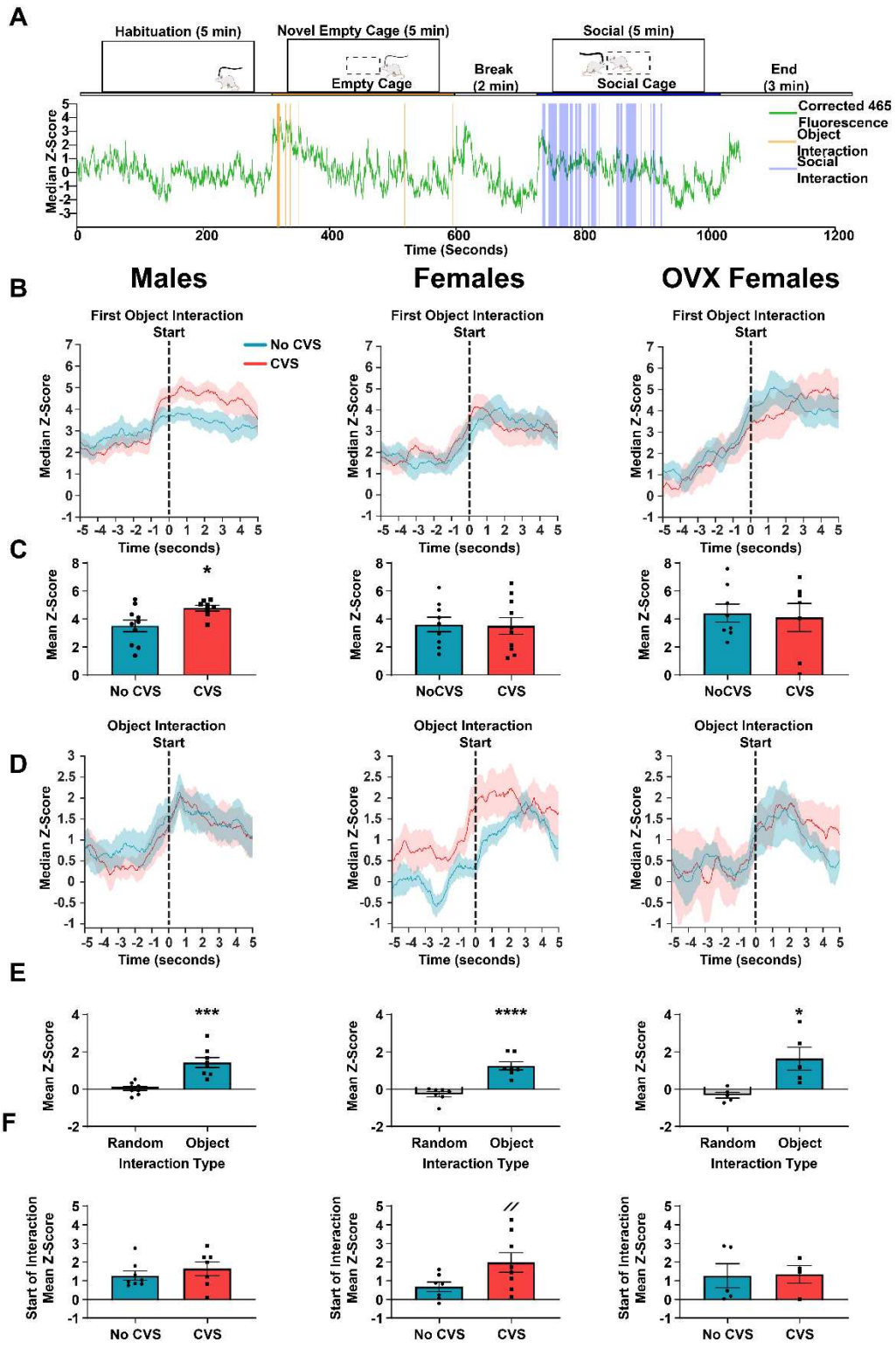
A
Habituation (5 min)
Novel Empty Cage (5 min)
Empty Cage
Break (2 min)
Social (5 min)
Social Cage
End (3 min)
Median Z-Score
Time (Seconds)
Corrected 465 Fluorescence
Object Interaction
Social Interaction
B
Males
Females
OVX Females
First Object Interaction Start
No CVS
CVS
Median Z-Score
Time (seconds)
C
Mean Z-Score
No CVS
CVS
NoCVS
D
Object Interaction Start
E
F
Random
Object
Interaction Type
Start of Interaction Mean Z-Score

Figure 9: IL glutamatergic neurons respond to object interactions. (**A**) Schematic of assay approach, with representative median-corrected 465 nm signal trace. (**B**) Group averaged z scores aligned to first object interaction, the dashed line represents interaction start. (**C**) CVS exposure increased mean signal during first object interactions in males, with no effect in females or OVX females. (**D**) Group averaged z scores aligned to object interactions. (**E**) All groups had higher mean Z-scores during object interactions compared to randomly selected non-interaction periods. (**F**) CVS trended ($p = 0.054$) towards increasing signal in females at the start of object interactions, with no effect in males or OVX females. Comparisons between treatments: // $p < 0.06$, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. OVX: ovariectomized.

Social Interactions

To determine how IL glutamatergic neurons encode social interactions, experimental rats were allowed to freely interact with a cage containing a novel conspecific. Social interactions were scored as nose pokes onto the empty cage. There were no effects of CVS on the mean signal during the first social interaction (data not shown). Photometry signal aligned to social interactions was averaged within animals then within groups (**Figure 10A**). Stress-naïve males, females, and OVX females all had higher mean signal during social interactions than random intervals (unpaired t-tests Random vs Social; Males: $n = 8$ $t_{(14)} = 5.3$ $p < 0.0001$, Females: $n = 9$ $t_{(16)} = 5.28$ $p < 0.0001$, OVX Females $n = 7$ $t_{(12)} = 8.05$ $p < 0.0001$ **Figure. 10B**). When comparing subsequent social interactions, excluding the first novel interaction, CVS reduced the mean signal during interactions in males and OVX females (unpaired t-tests No CVS vs CVS; Males: $n = 8$/group $t_{(14)} = 2.19$ $p < 0.05$, OVX Females $n = 7$/group $t_{(12)} = 3.09$ $p < 0.01$ **Figure. 10C**), but not intact females. Further, there was no effect of CVS on the mean signal during the first half-second of social interactions in either intact or OVX females, while in males stress-exposed animals trended towards lower than stress-naïve males (unpaired t-test CVS vs No CVS $n = 9$ $t_{(16)} = 2.06$ $p = 0.056$ **Figure. 10D**). My previous research determined that stimulation of IL glutamatergic neurons was sufficient to increase social motivation only in males; however, these results show that IL glutamatergic neurons have higher activity during social interaction

regardless of sex. To determine IL glutamatergic activity during the lead-up to social interaction, social interactions were filtered to have a relatively long period before interaction (>10 seconds, **Figure. 10F**). In males, the peak signal increased before the start of social interaction (Males mixed-effects model Time $F_{(2.648, 32.43)} = 5.2$ $p < 0.01$ **Figure. 10E**), which CVS reduced ($p < 0.05$), while there was no time-dependent change in signal in either intact or OVX females prior to social interaction. Overall, IL glutamatergic neurons had increased activity on average during social interactions, CVS reduced this activity in males and OVX females, and male IL glutamatergic activity increased prior to social interaction.

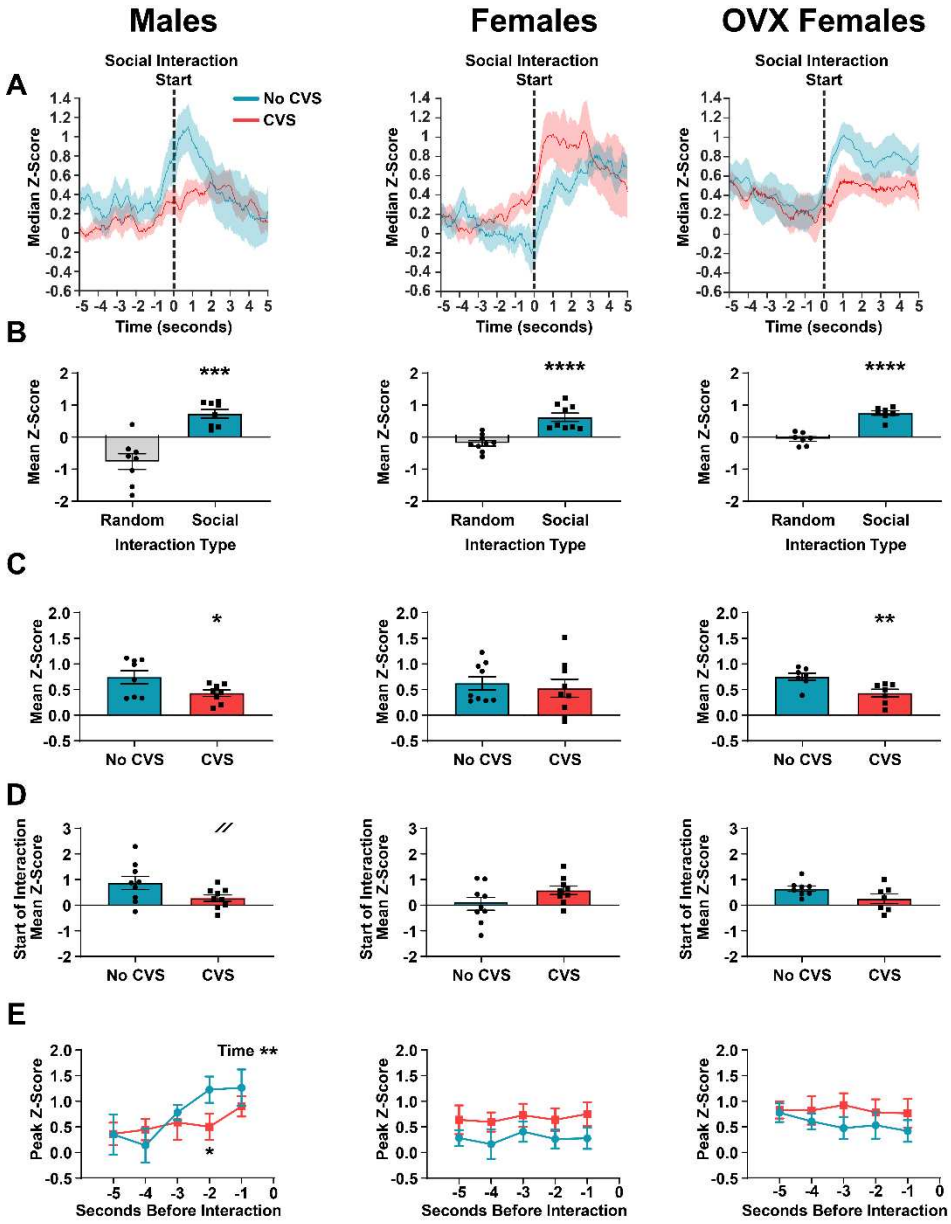

Figure 10: CVS reduces IL glutamatergic response to social interactions in males and OVX females. (**A**) Group averaged z scores aligned to social interactions, the dashed line represents interaction start. (**B**) All groups had higher mean Z-scores during object interactions compared to randomly selected non-interaction periods. (**C**) CVS exposure reduced mean Z-Score during social interactions in males and OVX females, with no effect in cycling females. (**D**) CVS trended towards reducing signal in males at the start of social interactions ($p = 0.056$), with no effect in females or OVX females. (**E**) Male's signal increased before social interactions with 10 seconds or longer of non-interaction time preceding them, CVS reduced this at 2 seconds prior to the interaction. Females and OVX females showed no change in signal preceding interaction. Comparisons between treatments: // $p < 0.06$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. OVX: ovariectomized.

Restraint

To determine the response of IL glutamatergic neurons to stressful stimuli in real-time as well as possible changes in this response to repeated presentation, rats underwent 30 minutes of restraint stress for 3 consecutive days. At the end of restraint stress, blood samples were collected to assess glucose, a measure of sympathetic-adrenal-medullary axis activation, and corticosterone, the product of hypothalamic-pituitary-adrenal axis activation. In males, CVS increased corticosterone, with the second and third days being higher than No CVS following post hoc comparisons (mixed-effects model: Stress $F_{(1, 27)} = 11.99$, $p < 0.01$, **Figure. 11B**). In cycling females there was an interaction effect between day and CVS (repeated-measures 2-way ANOVA: Day x Stress $F_{(2, 32)} = 3.57$, $p < 0.05$), CVS increased corticosterone response on day 1 in post hoc comparisons ($p < 0.05$). Similarly, OVX females had an interaction between day and CVS (mixed-effects model: Day x Stress $F_{(2, 39)} = 4.66$, $p < 0.05$, **Figure. 11B**), with CVS OVX females having higher corticosterone on day 1 ($p < 0.01$). Further, OVX No CVS females had higher corticosterone on day 2 compared to day 1 ($p < 0.05$). On day 1 cycling females had higher corticosterone response than OVX females, consistent with previous research (unpaired t-tests Female vs OVX Female n = 9-11/group $t_{(18)} = 2.76$ $p < 0.05$, Viau and Meaney, 1991). In males, there were overall effects of CVS and day on glucose response, with CVS exposed animal having higher glucose on days 2 and 3 following post hoc comparisons (mixed-effects model: Day $F_{(1.588, 39.69)} = 4.912$, $p < 0.05$, Stress $F_{(1, 28)} = 5.13$, $p < 0.05$, **Figure. 11C**). In comparisons with day 1, No CVS males had lower glucose on day 3 compared to day 1 ($p < 0.01$). In both intact and OVX females there was an effect of restraint day on glucose (mixed-effects model: Females Day $F_{(1.882,31.05)} = 13.66$, $p < 0.0001$, OVX Females Day $F_{(1.887,33.97)} = 4.43$, $p < 0.05$), with No CVS cycling females having higher glucose on day 2 compared to day 1 ($p < 0.05$).

Neural activity was measured as transient peaks using a rolling 1-minute median and MAD Z-score. Transients were defined as values above 2.91 (Gunaydin et al., 2014; Muir et al., 2018). On all days, across males, females, and OVX females there was a main effect of restraint increasing transient frequency. On day 1 in males, No CVS animals had higher transients in restraint but CVS animals did not (mixed-effects model: Restraint $F_{(1, 13)} = 19.50$, $p < 0.001$ Sidak's No CVS: $p < 0.0$, **Figure. 11D**). On day 1 both females and OVX females, restraint transient Hz was higher in restraint compared to home cage in No CVS and CVS animals (mixed-effects model: Females Restraint $F_{(1, 15)} = 36.35$, $p < 0.0001$ Sidak's No CVS: $p < 0.001$ CVS: $p < 0.01$; OVX females Restraint $F_{(1, 16)} = 70.07$, $p < 0.0001$ Sidak's No CVS: $p < 0.0001$ CVS: $p < 0.0001$). There were no differences between male, female, and OVX female transient Hz in restraint on day 1 (one-way ANOVA n = 7-9 $F_{(2, 20)} = 2.362$, $p = 0.12$).

On day 2 there was a main effect of restraint in males and OVX females, but no group-specific effects (mixed-effects model: Males Restraint $F_{(1, 17)} = 7.25$, $p < 0.05$, OVX Restraint $F_{(1, 18)} = 5.92$, **Figure. 11E**). Females, No CVS and CVS, had higher transient Hz compared to home cage (mixed-effects model: Restraint $F_{(1, 15)} = 36.35$, $p < 0.0001$, No CVS $p < 0.001$, CVS $p < 0.05$). On day 3 there was a main effect of restraint in males, with the No CVS group being higher than home-cage (mixed-effects model: Males Restraint $F_{(1, 19)} = 20.16$, No CVS $p < 0.001$, **Figure. 11F**). In cycling females there was a main effect of restraint, with No CVS animals having higher transient Hz in restraint compared to home cage (mixed-effects model: Restraint $F_{(1, 16)} = 24.85$, $p < 0.001$, No CVS $p < 0.001$). In OVX females there was a main effect of restraint, with CVS animals having higher transient Hz in restraint compared to home cage (mixed-effects model: Restraint $F_{(1, 17)} = 14.56$, CVS $p < 0.01$).

To assess the association between IL glutamatergic neural activity and physiological stress responses, Pearson's correlations were run between mean restraint transient Hz and glucose or corticosterone (**Table 4**). On day 1 there was a significant negative correlation between transient Hz and glucose in CVS exposed males. In No CVS males, on day 2 there was a significant positive correlation between transient Hz and glucose, and on day 3 significant positive correlations between transient Hz with glucose and corticosterone. There were no significant correlations between restraint transient Hz and glucose or corticosterone in females or OVX females.

Overall, CVS increased male glucose and corticosterone responses on the second and third days of restraint but only increased corticosterone on day 1 in cycling and OVX females. Further restraint increased activity overall in all groups across all days, but in males this effect was driven by the No CVS group.

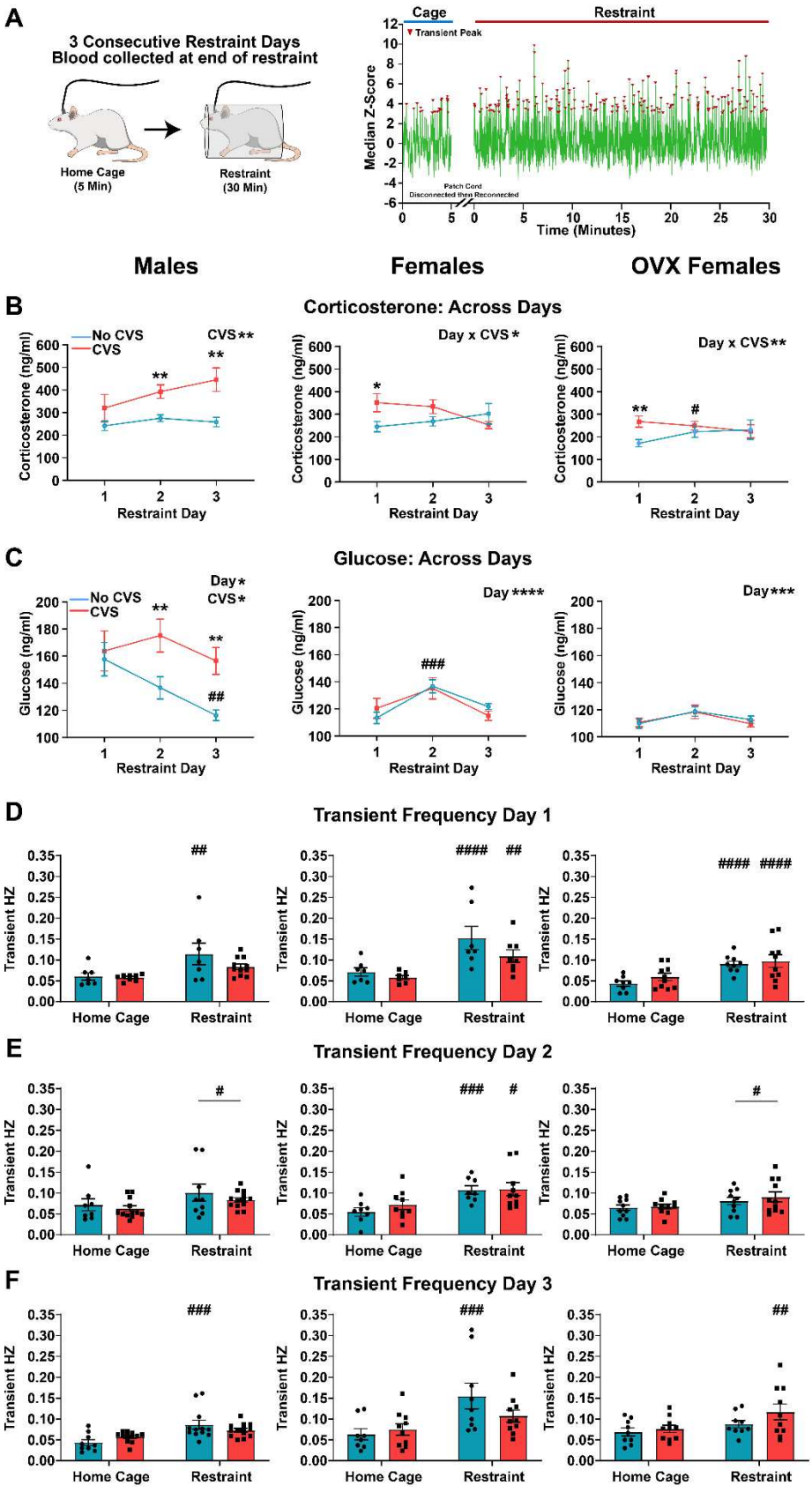
A
3 Consecutive Restraint Days
Blood collected at end of restraint
Home Cage
(5 Min)
Restraint
(30 Min)
Cage
Restraint
Transient Peak
Median Z-Score
Patch Cord
Disconnected then Reconnected
Time (Minutes)
Males
Females
OVX Females
B
Corticosterone: Across Days
No CVS
CVS
CVS**
Day x CVS*
Day x CVS**
Corticosterone (ng/ml)
Restraint Day
C
Glucose: Across Days
Day*
CVS*
Day****
Day***
Glucose (ng/ml)
Restraint Day
D
Transient Frequency Day 1
E
Transient Frequency Day 2
F
Transient Frequency Day 3
Transient HZ
Home Cage
Restraint

Figure 11: IL glutamatergic neurons are stress responsive. (**A**) Left: Schematic of restraint approach, Right: Representative transient peak quantification during home cage and restraint. (**B**) CVS increased corticosterone response in males on days 2 and 3, and day 1 in females and OVX females. (**C**) CVS exposure increased male glucose stress response on days 2 and 3. There was no CVS effect in females or OVX females. (**D**) Day 1 Left: Males No CVS animals had higher transient Hz during restraint than home cage. Middle: Both No CVS and CVS females had higher transient Hz during restraint than home cage. Right: No CVS and CVS OVX females had higher transient Hz during restraint than home cage. (**E**) Left and Right: There was a main effect of restraint increasing transient Hz in males and OVX females. Day 2 Middle: Both No CVS and CVS females had higher transient Hz during restraint than home cage. (**F**) Day 3 Left: No CVS Males had higher transient Hz during restraint than home cage Middle: Female No CVS animals had higher transient Hz during restraint than home cage, Right: OVX CVS females had higher transient Hz during restraint than home cage, Stress comparisons * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Within-stress time comparisons # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$. OVX: ovariectomized.

Table 4: Pearson's correlation coefficients of daily restraint activity with glucose and corticosterone.

		Males				**Females**				**OVX Females**			
		Glucose		**Corticosterone**		**Glucose**		**Corticosterone**		**Glucose**		**Corticosterone**	
Day	**Group**	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
1	No CVS	0.60	0.11	0.45	0.26	-0.60	0.12	0.30	0.46	-0.21	0.59	0.24	0.54
1	CVS	-0.63	0.04	-0.56	0.09	-0.13	0.75	-0.42	0.30	-0.49	0.15	-0.07	0.85
2	No CVS	0.66	0.04	0.48	0.16	-0.03	0.94	0.14	0.72	-0.25	0.48	0.54	0.11
2	CVS	0.49	0.07	-0.14	0.64	0.25	0.49	-0.39	0.26	0.53	0.10	-0.04	0.91
3	No CVS	0.71	0.01	0.84	0.001	0.58	0.10	-0.22	0.58	0.07	0.86	0.39	0.30
3	CVS	0.32	0.29	0.26	0.39	-0.19	0.65	-0.02	0.97	-0.21	0.56	-0.28	0.43

Chocolate

To assess IL glutamatergic neural response to food reward, animals were given a chocolate chip in their home cage. To reduce time to consumption and allow for recording, animals were given a chocolate chip on the night following the final restraint, and then twice

daily for the next two days. Animals were also habituated to home cage recording conditions for 15 minutes a day for two days before testing. To assess the role of caloric deficit in IL glutamatergic encoding of food reward, a cohort of food-restricted males was run. Food-restricted males underwent the same injections and cannulations as all other cohorts (**Figure. 12A**). Food-restricted animals had weight loss over two weeks similar to CVS-exposed rats. CVS and food-restricted animal bodyweight changes from the start to the end of treatment were different from No CVS (one-way ANOVA n = 7-14/group, $F_{(2,26)} = 11.07$, $p < 0.001$, Tukey post hoc comparisons) but not from each other (**Figure. 12B**). Photometry signal was aligned to rats grabbing chocolate reward (**Figure. 12C).** All No CVS groups had higher signal during the first half-second of grabbing the chocolate than randomly selected periods (unpaired t-tests random vs chocolate: Males: n = 7/group $t_{(12)} = 3.55$ $p < 0.01$, Females: n = 9/group $t_{(16)} = 5.154$ $p < 0.0001$, OVX Females: n = 6/group $t_{(10)} = 2.664$ $p < 0.05$, **Figure. 12D).** Intact No CVS females had higher signal to grabbing the chocolate than No CVS males or OVX females (one-way ANOVA n = 6-9 $F_{(2,19)} = 3.822$ $p < 0.05$**).** CVS exposed and Food-restricted males had higher signal to grabbing the chocolate than No CVS (one-way ANOVA n = 7-11 $F_{(2,20)} = 3.466$ $p = 0.05$). There were no effects of CVS in females or OVX females. Overall, all groups showed increased IL glutamatergic activity during food reward acquisition and CVS increased this response in males, an effect that is mimicked by food-restriction.

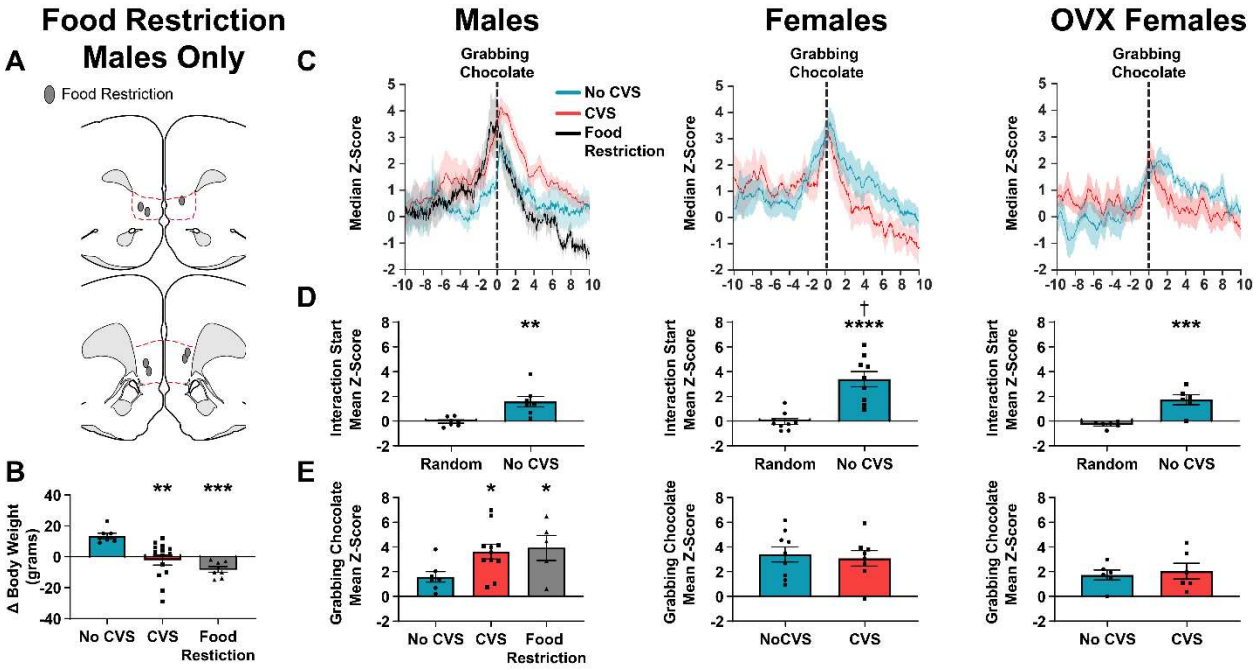

Figure 12: CVS and food-restriction increase male IL glutamatergic response to food reward. (**A**) Mapped fiber-optic cannula positions within or around the IL (red outline). (**B**) Food-restriction led to weight loss similar to CVS. (**C**) Group averaged z scores aligned to animals grabbing chocolate, dashed line represents rat grabbing chocolate. (**D**) All groups had higher mean Z-scores during the first half-second of grabbing the chocolate compared to randomly selected non-interaction periods. Cycling females had higher response than males or OVX females. (**E**) CVS exposure and food-restriction increased mean start Z-Score in males, with no effect in females or OVX females. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Female Mean z-score vs males and OVX females † $p < 0.05$**.** OVX: ovariectomized.

Discussion

The current study combined fluorescent calcium imaging of glutamatergic IL pyramidal neurons with behavioral and endocrine assessments. Across all stress-naïve groups, IL pyramidal neurons increased in activity during an object or social interaction, during an acute stressor, and food reward acquisition. However, the effects of chronic stress exposure were different between males and females, dependent upon both female ovarian hormone status and the behavior being tested.

Pharmacological, lesion and optogenetic studies have identified a role for the IL in regulating social behavior, stress responding, and reward processing (Wallace and Myers, 2021). Further, chronic stress leads to the accumulation of the long-term activation marker ΔFosB in the IL and alters aspects of IL neural signaling (McKlveen et al., 2016; Pace et al., 2020b; Wallace and Myers, 2021). However, the impacts of these signaling changes on overall IL excitatory-inhibitory balance have been mixed. Further, significant sex differences have been identified in the impacts of chronic stress exposure on IL neural function, with ovarian hormones modulating these differences (Shansky et al., 2010; Wei et al., 2014; Yuen et al., 2016). The mixed results of chronic stress effects on IL from slice and histological studies have made interpretations of stress impacts on IL neural activity during behavior difficult. Further, there are no studies to our knowledge recording the activity of genetically identified IL glutamatergic neurons. Thus, I sought to determine IL glutamatergic neural activity in real-time during behavioral testing as well as the effects of chronic stress exposure on this activity. Further, I sought to whether ovarian protect female IL glutamatergic neurons from the effects of chronic stress exposure. Altogether, I found that IL glutamatergic neural activity during behavior is similar in stress-naïve animals across males and females, regardless of OVX status, except for social approach and food reward acquisition. However, chronic stress primarily alters IL glutamatergic neural activity in males and not females, with some differences dependent upon ovarian status.

The object and social interaction test was performed to examine IL glutamatergic neural reactivity to novel non-social stimuli, and social stimuli. All groups showed increased activity during object interactions compared to random intervals, suggesting a role for IL neural activity in processing non-social stimuli. CVS increased male IL glutamatergic neural activity during the first object interaction, but not subsequent interactions, suggesting CVS alterations to IL

glutamatergic neural activity are specific to novelty. All stress-naïve groups increased IL activity during social interactions compared to random intervals. However, CVS exposure reduced IL glutamatergic neural activity during social interactions in males but not females. OVX females had a similar reduction in IL glutamatergic neural activity during social interactions as males, suggesting ovarian hormones are protective against CVS-induced changes to social processing in females. Interestingly, previous research has shown mPFC GABAergic parvalbumin (PV) neurons have increased activity during social interaction as well (Selimbeyoglu et al., 2017). Interestingly, my results demonstrate that IL glutamatergic neurons have increased activity on average during social interaction. Taken together, it appears that both inhibitory and excitatory pyramidal neurons within the mPFC increase in activity during social behavior. How the activity of these two functionally opposed cell groups work in network to modify both the quantity and quality of social interaction requires more work recording from both populations simultaneously. Further, males regardless of CVS showed increased activity in the lead-up to social interaction, which CVS reduced, while females and OVX females did not. These findings are in line with previous stimulation work where optogenetic stimulation of IL glutamatergic neurons increased social motivation in males but not females (Wallace et al., 2021). Taken together, these results demonstrate IL glutamatergic neurons participate in generating social approach behaviors in males but not in females. Overall, these results are the first *in vivo* demonstration that IL glutamatergic neurons have increased activity during object and social interactions regardless of sex. Further, chronic stress increased male response to the first interaction with a novel object, and reduced response to social interaction in males and OVX females.

The IL is stress-responsive, as identified histologically, and a component of the central autonomic nervous system (Sood et al., 2018; McKlveen et al., 2019). As such, neural signaling from the IL can alter aspects of endocrine and autonomic stress responses. In males, IL glutamatergic output is necessary for constraining the physiological stress response (Myers et al., 2017; Schaeuble et al., 2019). Further, optogenetic stimulation of male IL glutamatergic neurons is sufficient to reduce both the corticosterone and glucose response to restraint stress, though it facilitates glucose release in females (Wallace et al., 2021). Thus, in males IL glutamatergic neural activity is necessary and sufficient for the constraint of the acute stress response. Yet, though there are increases in immediate-early gene expression following acute stressors in the IL, these effects show sex-based differences and the time dynamics were unknown (Sood et al., 2018). My results are the first *in vivo* demonstration that vmPFC glutamatergic neurons significantly increase their activity in response to acute stressors. Further, in males CVS reduced IL glutamatergic neural response to restraint compared to home cage while increasing measures of the physiological stress response. Taken with the previously demonstrated role for IL glutamatergic neurons in constraining both the sympathetic and HPA stress responses, these results demonstrate that CVS blunts stress-induced increases in male IL glutamatergic neural activity. Lessened IL glutamatergic output during stress then likely contributes to the hyper-stress responding observed following chronic stress exposure. Altogether, our restraint results demonstrate for the first time *in-vivo* that IL glutamatergic neurons are acutely stress reactive. Further, chronic stress lessens IL glutamatergic neural response to acute stress likely contributing to hyper-stress responding.

Rats were given access to a chocolate chip to assess IL glutamatergic neural participation in hedonic food reward. All groups showed increased neural activity when initially grabbing the

chocolate chip. In males, CVS increased IL glutamatergic neural response to grabbing the chocolate. Food-restricted male neural activity mimicked this increase, suggesting that caloric deficit underlies aspects of this CVS phenotype. Interestingly, OVX females have similar weight loss following CVS to males but do not show a similar change in IL response. Further, stress-naïve females had a higher response to food reward acquisition than males or OVX females, suggesting an increased role of female glutamatergic neurons in food-seeking behavior that is altered by ovarian hormone status. These results are consistent with previous research showing male IL neural activity changes in reward-seeking (Burgos-Robles et al., 2013; Moorman and Aston-Jones, 2015). Overall, IL glutamatergic neurons increase in activity during food reward acquisition and this activity is altered by CVS only in males, an effect partially explained by caloric deficiency.

Utilization of GCaMP6s in the current study allowed for the first *in vivo* recording of IL glutamatergic neural activity through multiple behavioral tests and across biological sex. However, fluorescent indicators of neural activity are limited by their relatively slow kinetics in fluorescent responses to neural activity. While previous studies have demonstrated that photometry recordings are reflective of neural action potentials, the kinetics of GCaMP inherently cannot capture all action potentials (London et al., 2018). Yet, while more temporally specific recordings of neural activity would likely reveal further effects, no current method exists to directly record the activity of genetically-identified neural populations electrically *in vivo*. A further limitation is that our recordings were targeted to glutamatergic neurons broadly within the IL; however, this population has heterogeneous projection targets. As specific vmPFC projections can give rise to different, even contrasting, behaviors, recording activity of

projection-defined populations would provide clarity on the observed sex differences and chronic stress effects.

Altogether this study provided the first *in vivo* recordings of IL glutamatergic neural activity across males and females. Our results found minor sex differences in responses to social approach and food reward acquisition. However, chronic stress exposure primarily altered male but not female IL glutamatergic neural activity. Importantly CVS exposure did not strictly increase or decrease male IL glutamatergic neural activity as previously hypothesized, instead it altered activity differently depending on the specific behavioral context. Thus, the effects of chronic stress exposure on IL glutamatergic activity are highly context-dependent rather than discrete hyper- or hypo-inhibition. These results suggest that new antidepressant treatments should not focus on broadly altering cortical excitability, instead they must focus on correcting altered circuit activity patterns within specific behavioral contexts. These treatments will need to account for not only the specific behavior being corrected, but also individual patient variables such as biological sex and variations in gonadal hormones. While this novel *in vivo* recording study has provided groundwork to understand prefrontal activity patterns, more work is required to determine how specific prefrontal neural populations respond to and regulate behavior and physiology.

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CHAPTER 5: CONCLUSIONS

Dissertation Scope and Contributions

The overarching goal of the work in this dissertation was to address limitations in our understanding of the neural regulation of depression-associated behaviors. Currently, our understanding of depression is limited by our incomplete knowledge of how genetically-specific neural populations within the ventromedial prefrontal cortex (vmPFC) regulate behavior. To address this limitation, in Chapter 2, I identified that stimulation of glutamatergic vmPFC neurons imposes a positive valence, increases social motivation, and constrains the acute stress response in adult male rats. While in adult female rats, the stimulation of the same neural population does not alter affect or social motivation and enhances the acute stress response. Following the unexpected sex-based differences observed in chapter 2, I utilized a circuit approach to dissect how a specific vmPFC to hypothalamic projection contributes to the previously observed behavioral and physiological phenotypes. I discovered that stimulation of vmPFC glutamatergic output in the posterior hypothalamus generates positive affective valence in both sexes but does not alter social motivation. Further activation of this circuit constrains the sympathetic stress response in males but augments the HPA stress response in females. Altogether, stimulation work identified substantial sexual divergence in prefrontal regulation of behavior and acute stress-responding; further, it identified a specific neural circuit that accounts for aspects of these sex differences. In Chapter 4, I sought to determine the activity of vmPFC neurons during behavior and how this activity is disrupted in a preclinical model of MDD, chronic stress exposure. I found that vmPFC glutamatergic activity is similar in males and females, with differences in social approach and reward processing. However, chronic stress

exposure altered vmPFC glutamatergic neural activity primarily in males, with ovarian hormones protecting females against some chronic stress effects. Overall, the work outlined in this dissertation identified significant sexual divergence in the regulatory capacity of vmPFC neurons in behavioral and physiological functions, as well as in the effects of chronic stress on neural activity.

Neural Activity Patterns

One of the major goals of this dissertation was to address the lack of studies focused on recording prefrontal neural populations, specifically in genetically-defined neural populations and in female subjects. Previous preclinical depression research has been manipulating prefrontal function and assessing behavioral or physiological phenotypes. However, without recording neural activity pharmacological studies produce results that are difficult to interpret. For example, investigations of antidepressants in rodents have identified that applications of both selective serotonin reuptake inhibitors as well as the more novel rapid-acting antidepressant ketamine increase histological markers of neural activity in the vmPFC (Chang et al., 2015; Fuchikami et al., 2015; Bentefour et al., 2016). However, while these studies demonstrate that these compounds alter vmPFC activity, the precise nature of these changes is unknown. Further, it is unclear how to incorporate histological indicators of neural activity into our understanding of endogenous vmPFC neural signaling during behavior. Unfortunately, the degree and length of neural activity required for the expression of histological markers of activity is not well characterized.

The reliance on histological markers of neural activity has made interpretations of neural regulation of behavior difficult, as they do not provide information about specific neural activity patterns. A similar limitation is present in human literature. Due to the necessity of non-invasive methods of recording neural activity, most clinical recording studies utilize changes in blood flow or glucose metabolism as their measure of changes to neural activity patterns (Kim et al., 2021). However, these methods cannot record the activity of specific neural populations and often cannot capture rapid changes in neural activity. Addressing these limitations will require shifting the primary focus of preclinical research from manipulation-based to broad recordings of neural activity focused on both genetic and projection specificity. To address these limitations in the context of a depression-associated neural population, I performed the first *in vivo* recordings of vmPFC glutamatergic neurons across a broad range of behavioral tests. While this work has limitations, my findings can provide context to future studies manipulating vmPFC neural activity. Further, the findings of these novel *in vivo* genetically targeted recordings of neural activity demonstrate the necessity of understanding how specific neural population activity patterns are affected by physiological state, specific behavioral context, and biological sex.

One of the most limited aspects of our understanding of prefrontal neural function is the specific timing of neural activity patterns. This limitation exists in both our understanding of endogenous activity patterns as well as the activity patterns induced by pharmacological manipulations. For example, within my stimulation of vmPFC neurons, I found the importance of stimulation frequency on behavioral regulation. In Chapter 2, stimulation of vmPFC neurons at 10 Hz increased social motivation in males, but 20 Hz stimulation did not. Further my recordings in chapter 4, showed that vmPFC glutamatergic neurons increased in activity before social interaction only in males. Taken together, these results suggest that changes in male

vmPFC glutamatergic neural activity both signal and regulate social approach; however, overstimulation of these neurons disrupts this function. These results demonstrate the importance of understanding how neural populations endogenously encode behavior to tune manipulations within physiological activity patterns. However, currently due to the lack of *in vivo* cell-type specific recordings of neural activity it is difficult to appropriately target stimulation parameters.

The specificity of stimulation parameters is particularly relevant in the manipulation of specific projection-defined circuity. For example, our goal with stimulation of the vmPFC to PH circuit in chapter 3 was to broadly examine the regulatory capacity of vmPFC influence in the PH. However, while our stimulation was tied to endogenous firing rates of prefrontal neurons at 10 Hz, there are no studies examining the specific activity patterns of this circuit. Thus, without information regarding vmPFC to PH signaling, it was impossible to target our stimulation beyond broadly increasing activity to determine behavioral regulation. While our stimulation demonstrated some of the regulatory capacity of this circuitry, it is likely that vmPFC neurons can regulate other aspects of behavior through the PH but require more specific activity patterns to do so. This specificity of phenotypes based on the parameters of stimulation has been demonstrated previously. Hyosang Lee et al. demonstrated that neurons in the ventromedial hypothalamus generate mounting or aggressive behaviors depending upon light intensity (Lee et al., 2014). Thus, understanding how neural circuits endogenously respond to a variety of contexts is paramount to properly manipulating that neural circuitry. Recordings of circuit-specific neural activity throughout different contexts would provide the necessary information to allow for physiologically tuned manipulations. These manipulations could then determine the contribution of prefrontal circuit activity patterns to behavioral and physiological regulation. The aggregation of studying these activity patterns and their necessity could then be utilized for

exploring human prefrontal circuitry. While some of the circuit activity patterns will be specific to rodent cortical function, the discovery and categorization of generalized prefrontal circuit motifs would greatly assist in understanding prefrontal function.

Altogether, current limited recordings of cell-type-specific neural populations has made understanding neural signaling of behavior difficult. Without this necessary information about endogenous neural activity patterns, understanding how current treatments alter neural activity to ultimately influence behavior has been impossible. Further, lack of recording information has made even stimulation of specific cell-types difficult to interpret. Without tuning stimulation of neural populations within their endogenous activity patterns, stimulation studies can only state what neurons can regulate but cannot determine what behaviors these neurons endogenously regulate. While work remains, my stimulation and recordings of a specific vmPFC neural population have addressed these limitations by providing information about what behaviors and processes this population can regulate and demonstrating the endogenous activity of this population during these behaviors.

Behavioral and Physiological integration

Another outcome of the work presented in this dissertation is the intertwined nature of prefrontal function and physiological state. Historically, behavioral, and physiological outcomes have been separated when considering both clinical and preclinical experimentation. However, increasing evidence indicates that mood disorders are strongly tied with physiological dysfunction. Indeed, there is strong comorbidity between MDD and a variety of physiological diseases including metabolic, cardiovascular and respiratory diseases, as well as obesity (Ghanei Gheshlagh et al., 2016; Goldstein et al., 2019; Patsalos et al., 2021; Sakharkar and Mai, 2021). In

the above work, I demonstrated that a subset of vmPFC neurons can regulate both affective state, social motivation, and physiological responses. I also found that the activity of these neurons is altered by chronic stress and physiological state such as caloric deficit. Thus, develop more complete understanding of prefrontal function we must determine how alterations in physiological state interact with prefrontal neural activity patterns.

As outlined in the introduction, gonadal hormones such as estradiol can influence prefrontal function and alter the effects of chronic stress (Hao et al., 2006; Delevich et al., 2021; Wallace and Myers, 2021). However, though the effects of estradiol on phenotypic outcomes are so pervasive, its influence on prefrontal neural function is not well understood. Ovarian hormones have a demonstrated role in altering the development of the prefrontal cortex (Premachandran et al., 2020; Delevich et al., 2021); however, they can also acutely alter prefrontal neural excitability (Yousuf et al., 2019). Yet, the influence of ovarian hormones on the activity of specific cell-types of the prefrontal cortex *in vivo* is not known. However, a recent publication outlines a study design to better determine how ovarian hormone fluctuations interact with neural activity patterns. Clemens et al. performed an in-depth experiment in which they recorded the activity of somatosensory neurons to repeated presentations of facial touch in female rodents. At the same time, they monitored the estrus phase of the female rodents, they could then correlate changes in the activity patterns of somatosensory neurons to facial touch with fluctuations in ovarian hormones. They concluded that the firing activity of fast-spiking interneurons within the somatosensory cortex to facial touch was tightly coupled to changes in circulating estradiol (Clemens et al., 2019). While the conclusions from this work are specific to the somatosensory cortex, the study design should be broadly replicated. Principally, recording cell-type specific activity in conjunction with repeatable trial-based stimuli and consistent

physiological monitoring. Similar studies of prefrontal neural circuit function would greatly advance our understanding of how prefrontal circuitry is altered by fluctuations in ovarian hormone levels. However, these studies need to be further expanded to include a variety of physiological factors. The list of physiological factors that influence prefrontal function comprises the majority of signaling molecules within the body, including leptin, melatonin, cortisol, testosterone, progesterone, and insulin (Ge et al., 2018; Killgore et al., 2018; Trujeque-Ramos et al., 2018; Zou et al., 2019). Yet, how these factors influence the activity patterns of prefrontal neural population *in vivo* and in real time is not known. By performing in-depth recording studies examining how these factors influence prefrontal neural activity, we can dissect how physiology interacts with prefrontal circuits and ultimately changes behavior.

Overall, limited knowledge of how specific neural populations within the prefrontal cortex regulate behavior in real time has restricted our ability to develop effective treatments of MDD. In this dissertation, I demonstrated considerable sex differences in the regulatory role of a specific vmPFC neural population. However, treatments focused on the human homologous region BA25 have not addressed sex differences or the possible interactions of ovarian hormones in treatment efficacy. This work demonstrates a critical need to understand and study prefrontal pathology differently based on sex. Further, I have elucidated mechanisms through which environmental and circulating factors alter neural population activity, providing possible targets for future therapeutics to correct behavioral and physiological dysfunction. However, considerable work remains to ensure that studies are aligned to best determine how endogenous neural circuit activity regulates behavioral output. In this final chapter, I have outlined the current limitations within the field in understanding prefrontal neural regulation of behavior. Ultimately, preclinical studies require a largescale increase in recording neural activity patterns

to better understand how endogenous circuits function, and how these circuits can be manipulated to alter behavior. By greatly expanding our knowledge of neural activity patterns, we can determine what circuit activity is specific to preclinical models and what patterns can be carried forward to human clinical treatments. These large-scale neural recording studies would not only greatly enhance our knowledge of the mechanistic relationship between the brain and behavior but would also provide more specific targets for developing novel effective MDD therapies.

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APPENDIX

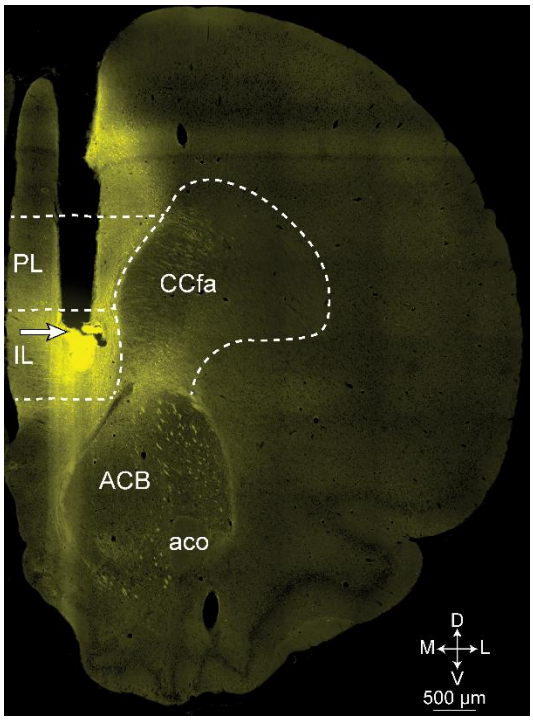

Figure S1: Photomicrograph of IL cannulation site. 10x image of cannula tract targeting the IL with AAV-delivered YFP fluorescence. White arrow indicates location of fiber tip. Dashed white lines indicate region borders. ACB - nucleus accumbens, aco – anterior commissure, CCfa – corpus callosum anterior forceps, IL – infralimbic, PL- prelimbic.

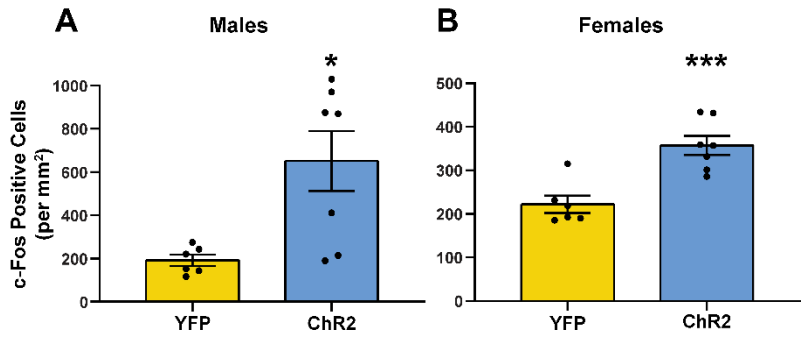

Figure S2: Optogenetic stimulation increased c-Fos positive cell count in males and females. At the conclusion of experiments 2 and 3, rats received 5 minutes of optic stimulation (10 Hz, 5 ms pulses, 1 mW) followed by 90 minutes of recovery prior to tissue collection. Chromogen immunohistochemistry was used to identify and quantify c-Fos positive cells in the 16 IL. * $p < 0.05$, *** $p < 0.005$ vs YFP within sex.