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GPR171 Modulates Mood, Consummatory Behaviors, And Morphine Antinociception in a Sexually Dimorphic Pattern in Mice

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GPR171 MODULATES MOOD, CONSUMMATORY BEHAVIORS, AND MORPHINE ANTINOCICEPTION IN A SEXUALLY DIMORPHIC PATTERN IN MICE

by

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ABSTRACT

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Megan C. Raddatz, Doctor of Philosophy

Utah State University, 2024

Major Professor: Dr. Erin N. Bobeck
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G Protein Coupled Receptors (GPCRs) are one of the most druggable classes of receptors due to their known involvement in a wide variety of biological processes, diseases, and disorders. GPR171, a recently deorphanized GPCR, is of particular interest as a therapeutic target, due to the recent development of a functional agonist and antagonist. GPR171 has proposed roles in mood, feeding, and pain regulation, however, all studies thus far have been limited to using pharmacological manipulation with drugs or a restrictive viral knockdown method. Additionally, studies on GPR171 have largely neglected using female mice as subjects. As such, sex differences in receptor actions are unclear. In this dissertation, we use pharmacological techniques to explore the role of GPR171 in females in mood, in order to directly compare to previous literature. We also use a newly developed GPR171 knockout mouse to directly explore the necessity of GPR171 in a variety of behaviors, including feeding, physiological functions, mood, and

pain, thus overcoming the limitations of traditional pharmacological approaches, while directly comparing males and females. In the first study, females given the GPR171 antagonist displayed decreased anxiety-related behaviors, similar to previous research with male mice. However, this effect was abolished in mice that underwent stress. Additionally, GPR171 actions and expression were sensitive to estrogen, representing a potential explanation of previously explored sex differences. In the second study, GPR171 knockout mice displayed a variety of altered behaviors relative to wildtype mice, including ataxia, alterations in feeding, anxiety, and depression-like behaviors. These effects were found to largely depend on interactions between sex and genotype. Finally, in the third study, genetic deletion of GPR171 reduced the efficacy of morphine antinociception, corroborating previous findings that used pharmacology. Collectively this dissertation confirms the critical roles of GPR171 and establishes that the receptor is not only sufficient to drive behavioral changes in mood, pain, and feeding, but is also necessary for these behaviors to occur normally. Importantly, this research also highlights the receptor's sexually dimorphic nature and actions, underscoring the need to consider sex-based differences as GPR171 continues to be evaluated as a therapeutic target for various diseases and disorders.

(114 pages)

PUBLIC ABSTRACT

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Receptors in the brain influence everything from complex behaviors related to mood, all the way to simple physiological functions like the way a person moves. Receptors are activated or inactivated by chemicals or hormones that the body produces or that are created to mimic the body's natural chemicals. Of the hundreds of receptors in the brain, GPR171 is particularly interesting because new drugs have been created to activate or block the receptor, and are being proposed for the treatment of different disorders, particularly disorders related to pain. GPR171 has been shown to affect pain behaviors, eating, and mood-related behaviors, but has not been well researched beyond these few studies. In addition, it is not clear what GPR171 does in females, as they have not been included in most of the research of the receptor. In our research, we explored how GPR171 affects females, particularly in anxiety, depression, and stress, to better understand its role. We also used a new method involving mice genetically modified to lack GPR171 to investigate its broader influence on behaviors such as eating, body functions, mood, and pain, directly comparing the results between male and female mice. Our findings show that blocking GPR171 in female mice reduced anxiety-like behaviors and was influenced by estrogen, suggesting that hormones may change how GPR171

works. We also show that mice without GPR171 displayed changes in eating behavior, movement coordination, anxiety, and depression, which were dependent on the sex of the mice. Additionally, these modified mice showed a reduced response to morphine, a common pain medication, confirming that GPR171 is necessary for morphine to work properly. Overall, this dissertation underscores the importance of GPR171 in controlling various behaviors and bodily functions. It shows that this receptor is essential for normal functioning and that its effects can differ significantly between males and females. This highlights the need to consider these differences as treatments targeting this receptor for various medical conditions are being developed.

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helped me weather the storms, and enjoy the sunshine.

This dissertation may bear my name as the sole author, but it truly is the culmination of the efforts of hundreds of individuals who have shaped, directed, and influenced me every day. I am profoundly grateful to everyone involved in my journey.

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ACRONYMS

GPCR	G Protein Coupled Receptor
EPM	Elevated Plus Maze
OFT	Open Field Test
FST	Forced Swim Test
RT-qPCR	Reverse Transcription-quantitative Polymerase Chain Reaction
BLA	Basolateral Amygdala
PFC	Prefrontal Cortex
NAc	Nucleus Accumbens
Hab	Habenula
vHipp	Ventral Hippocampus
KO	Knockout
WT	Wildtype
HET	Heterozygous
PAG	Periaqueductal Gray

CHAPTER I

INTRODUCTION

G protein-coupled receptors (GPCRs) are the most abundant and versatile class of receptors in the brain, implicated in nearly every physiological function, and heavily involved in psychological disorders. Of the 800+ GPCR members, approximately 140 remain in an orphan state with unknown ligands, while many others remain poorly characterized with unclear functions[1]. One poorly characterized GPCR, GPR171, is of particular interest because, while de-orphanized, its biological functions have only recently begun to be uncovered. GPR171 is activated by the endogenous peptide BigLEN, a cleavage product of the precursor peptide ProSAAS, which is the most abundant neuropeptide in the brain[2]. The receptor and its natural ligand are highly expressed in brain areas known to play various roles in emotional regulation, motivation, pain, and physiological homeostasis[2–4]. While there is significant potential for therapeutic targeting of GPR171 given its localization and the little research that has been conducted thus far, advances in our understanding of the neurobehavioral functions of this receptor and its ligand are required before serious considerations of therapeutics are further pursued. Understanding of GPR171/BigLEN's functions in both normal and pathological states remains limited and requires further investigation.

Primary Functions of GPR171

Given the limited information concerning GPR171, its functions have primarily been inferred from its expression patterns in the brain. GPR171 is most highly expressed

in the hypothalamus[2] which regulates internal states such as feeding and drinking. As a result, GPR171 has primarily been considered a receptor involved in feeding. In support of this hypothesis, male mice that received large doses of the endogenous ligand, BigLEN, ate and drank more compared to control mice[5], while inhibiting GPR171 decreased acute feeding[2]. While some studies have corroborated this positive correlation between GPR171 activity and feeding[2,3,6], other studies indicate that changes to the GPR171 system were negatively correlated with feeding[2] or did not alter feeding[3,7]. The most consistent effects of GPR171 on feeding were observed after a period of food deprivation[3,6,8], indicating that GPR171 is more involved in increasing motivation to adapt to feeding needs[9]. As such, while GPR171 modulates feeding, the effects appear more connected with affective drive rather than physiological need.

The evidence expanded to indicated that GPR171 also plays a role in affective regulation with the observation that, in addition to its expression in the hypothalamus, it is also highly expressed in the amygdala[3]. The amygdala, both central and basolateral areas, has well-established roles in regulating affective states and emotions. In male mice, systemic or intra-amygdala antagonism of GPR171 led to a decrease in anxiety measured in the open field test and elevated plus maze[3]. However, the GPR171 antagonist did not have any effects on depressive behaviors in the forced swim test despite the high comorbidity between anxious and depressive states[3]. Interestingly, exogenous agonist or BigLEN injections had no effect on anxious behaviors[8], while mice lacking the peptide precursor ProSAAS, displayed higher anxiety behaviors in the open field test, dark-light emergence test, and elevated plus maze[7]. Together, these studies indicate a role for GPR171 in anxious behaviors, although that role is dependent on whether ProSAAS,

BigLEN, or exogenous ligand levels were manipulated.

Beyond modulating feeding and anxious behaviors, recent studies have emphasized the potential role of GPR171 in modulating pain, both peripherally and centrally. ProSAAS levels are upregulated in the cerebrospinal fluid of fibromyalgia patients[10] and, upon treatment, decreased in patients with neuropathic pain[11]. ProSAAS levels were also altered in the cerebrospinal fluid of individuals with lumbar disk herniation[12]. While ProSAAS effects are possibly mediated through various receptors and cleavage products, evidence points towards direct involvement of GPR171 and BigLEN in mediating ProSAAS's role in pain. In chronic, inflammatory, and pathological pain, GPR171 agonism consistently decreased pain responses in male mice, though the timeline and efficiency differed between studies[8,13]. Additionally, in acute pain, despite not producing antinociception on its own, the GPR171 agonist had synergistic effects with morphine, leading to higher levels of antinociception than morphine alone[4]. Although more research is needed, given the supposed functions of GPR171, it holds promise as a therapeutic target for various pain, anxiety, and feeding pathologies. However, the understanding of its functions is still incomplete and complex, with a considerable degree of disparity and ambiguity in what is currently known.

Factors that Impact GPR171 Function

Several factors influence GPR171, adding to the complexity of the receptor's actions and functions. First, GPR171 has been shown to work in tandem with other receptors through receptor heterodimerization. GPR83 is a sister receptor that binds with PEN, a different ProSAAS derived peptide, and interacts directly with GPR171 to alter

the signal that either receptor produces alone[14]. In cell culture experiments, knock-out or knock-down of GPR171 affected the signaling of GPR83 and vice versa[14]. In addition, the receptors were discovered to be close enough in the hypothalamus of mouse brain slices to functionally interact. GPR171 has also been shown to functionally interact with the mu-opioid receptor[4]. While the consequences of these heterodimerization have yet to be explored, the ability of GPR171 to function in tandem with other receptors is a critical aspect of its function and may explain the synergistic effects of morphine and the GPR171 agonist in pain relief in mice[4].

Another crucial influence on GPR171 function that has received almost no attention in the literature is the impact of sex-differences. Surprisingly, every scientific result presented above, with the exception of the ProSAAS studies involving pain in humans[10–12], only applies to male mice. Two studies have looked at GPR171's actions in females and found there to be a sexually dimorphic response. First, while in male mice GPR171 agonism decreases chronic and neuropathic pain, this effect is absent in females[13]. A second study showed that there was synergy between morphine and GPR171 agonist treatments as documented by McDermott et al.[4], but it was to a smaller degree in female mice than in male mice[15]. While very little is known outside these results concerning the actions of GPR171 in females, estrogen has been shown to downregulate ProSAAS levels in a specific subset of neurons in the brain[16], and can directly influence levels of non-estrogen receptors in the brain[17] potentially mediating sex differences seen in these two GPR171 studies.

Finally, while many other factors influence the tone of ProSAAS and GPR171 and their functions, the potential impact of stress on GPR171 function deserves mention.

There are currently no studies evaluating the effect of stress on GPR171, but there is evidence that ProSAAS is downregulated in rats placed through a stress paradigm[18]. Neuropeptides not only participate in stress physiology but are also actively being considered as potentially clinically relevant components to the stress response[19]. Given GPR171's role in modulating acutely stressful states, such as pain or anxiety, it is highly probable that the receptor participates in the stress response over longer periods of time and is modulated by stress. Overall, the functions of GPR171 are likely complicated by many factors, but researchers have lacked the proper tools to directly assess the receptors functions as a whole and in consideration of the factors presented thus far.

Tools to Study GPR171

While significant advances in our understanding of GPR171's functions have occurred since its initial deorphanization, research has been conducted using pharmacological approaches following primarily systemic administration. While this gives a general idea of GPR171 drug actions, the endogenous functions of GPR171 remain unknown. The synthetic agonist and antagonist, while relatively selective for GPR171, are subject to unknown pharmacodynamic and pharmacokinetic regulation[3,6]. In addition, the drugs have unknown half-lives and lack the affinity of the endogenous ligand. The endogenous ligand, BigLEN, or BigLEN antibodies, have the greatest affinity, but are considered unstable when used *in vivo,* and it's unclear how effective they are when injected intracerebrally[2]. Alternatively, viral knock-down studies, while valuable, are only capable of assessing GPR171 actions in a limited area of the brain and have resulted in a 60% decrease in receptor levels, rather than complete knockout[3].

Finally, while a great deal of research has been conducted on ProSAAS, assessing the levels of ProSAAS or knocking out the peptide is an indirect measure of what may be happening through GPR171 or possibly through one of the other peptides cleaved from ProSAAS[20].

In addition to the methodological limitations, our understanding of how GPR171 works in females has been neglected in every study apart from two[13,15], despite a known sexual dimorphic response[13]. As such, the experiments in this thesis were conducted to fill the critical void in our understanding of GPR171's overall functions by exploring both sexes using the tools already in effect (Chapter 2) and using new tools, primarily that of GPR171 global knock-outs (Chapters 3 and 4). In so doing, we clarify the roles of GPR171 that have thus far been ambiguous or unclear due to methodological limitations, while expanding on that which has been neglected in the literature, further validating the pharmaceutical potential of GPR171 for the treatment of various disordered states.

CHAPTER II

THE GPR171-BIGLEN SYSTEM REGULATES ANXIETY BEHAVIORS IN FEMALE MICE AND IS INFLUENCED BY ESTROGEN

1. Introduction

Anxiety and depression are the most prevalent mental health disorders in the United States, constituting an escalating health crisis that impacts over 50 million individuals collectively[21,22]. While approximately two-thirds of those affected are female[23,24], there is a significant lack of information on the neural mechanisms and treatment of anxiety and depression within this demographic. However, it is evident that the interaction between gonadal hormones and stress reactivity underlies sex differences in the occurrence and treatment of mood disorders[25]. Given the rising prevalence of anxiety and depression, there is an urgent need for new and more effective treatments, with a particular emphasis on studying these disorders and their treatment in females.

Neuropeptides and their receptors offer promising targets for treating anxiety and depression. Many neuropeptides and their receptors are dysregulated in depression and anxiety[26], including ProSAAS and its derivatives which are the most highly expressed neuropeptides in the brain[18,27]. Consistent with these findings, systemic antagonism of GPR171, a receptor for one of the derivatives of ProSAAS (BigLEN), decreases anxiety behaviors in male mice[3]. Despite the promising effects of GPR171 antagonism in reducing anxiety, this has not been studied in females. However, it is clear that the action of GPR171 is not uniform between males and females, as agonist treatment relieves neuropathic and inflammatory pain in males, but not in females[13]. As such, while

GPR171 may be a promising pharmaceutical target for mood disorders in males, its efficacy in females is unknown.

Additionally, the interaction between GPR171 and stress has not been evaluated. The pathophysiology of anxiety and depression is driven by both the activity of receptors and ligands, and a dysregulated response to stress. The increased susceptibility to stress in females is thought to be a major contributing factor to their higher prevalence of anxiety and depression[28,29]. While there are no studies that directly evaluate the effects of stress on the BigLEN/GPR171 system, LittleSAAS, a separate derivative of ProSAAS that goes through the same cleavage process as BigLEN, was downregulated in rats exposed to a stress paradigm[18]. As such, the BigLEN/GPR171 system is also likely to be regulated by stress and manipulation of the system may modulate anxiety and depressive behaviors.

Here we explored GPR171 in anxiety, depression, and stress-related behaviors in females and associated changes in gene expression. To better understand GPR171's role in females we undertook three experiments: First, we injected female mice with a single injection of either the GPR171 agonist, antagonist, or vehicle and assessed depression- and anxiety-related behavior. Next, we injected female mice with the same set of drugs, 15 minutes prior to a stressor for six consecutive days, and assessed the effects of the subchronic stress and drug treatment on anxiety and mood-related behaviors. Lastly, we removed the ovaries from a set of female mice, controlling for endogenous estrogen levels and subjected the mice to the forced swim test in combination with either the GPR171 agonist or vehicle to assess for interactions between estrogen and GPR171 actions. We also analyzed GPR171 and ProSAAS gene expression to determine the

effects of stress and estrogen levels in brain areas related to anxiety and depression. Taken together, this study sought to further determine the role of GPR171 in mood, while considering interactions with stress and estrogen, and neural substrates.

2. Material and methods

2.1 Subjects

Subjects consisted of 133 female C57BL/6 mice (Charles River Laboratories, Wilmington, VA), aged 7-10 weeks, weighing 15-23g at the start of each experiment. Mice were group housed (four to five per cage), in a temperature and humidity-controlled room on a 12:12 light cycle (lights on at 0700). Testing took place between 0800 and 1800. Food and water were available *ad libitum* except during testing. Estrus cycle stage was confirmed via vaginal smear on the last day of each experiment. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health and were approved by the Utah State University Institutional Care and Use Committee.

2.2 Drugs

The GPR171 antagonist, MS21570 (5 mg/kg, i.p.; Cat. 6298 Tocris), and the GPR171 agonist, MS15203 (10 mg/kg, i.p.; as described in [15]) were diluted in 10% DMSO in saline (Cat. 67-68-5 Sigma-Aldrich). All drugs were administered at a volume of 10 ml/kg, 15 minutes prior to the start of experimental protocols. These doses and drug delivery time course were selected based on previous literature[3,4,13].

2.3 Behavior testing

Mice were habituated and handled for at least 2 days prior to each experiment. All behavioral testing was conducted by experimenters blind to the treatment groups. Equipment was cleaned with 70% ethanol after each test to eliminate any residual odors. Experiments were completed at the same time point each day to avoid temporal confounds. Drugs were administered 15 minutes prior to behavior testing.

2.3.1 Forced Swim Test. Mice were placed in a 4-liter glass cylinder with approximately 2500mL of water (24-26° C) for six minutes. Immobility, as defined by the absence of any movement other than those required to stay afloat, was recorded by two independent reviewers blind to the drug condition. Inter-rater reliability was calculated to ensure adequate agreement between reviewers (ICC > 0.85) and then scores were averaged across reviewers. Immobility during the final four minutes of each test was used as a measure of depressive behavior/behavioral despair[30].

2.3.2 Elevated Plus Maze (EPM). The EPM consisted of a platform with four arms – two with walls, two open – elevated approximately 40cm off the ground. Mice were placed in the center of the apparatus and allowed to freely explore for 15 minutes. The distance traveled, number of entries into each arm, time spent in each arm, and the percent of entries into the open arms were automatically calculated using ANYmaze software. The ratio of time spent in the closed arms vs. open arms was calculated as a measure of anxious behavior, with higher values indicating higher anxiety-like behaviors. By analyzing anxiety as a ratio of time in closed vs open arms, any potential differences

in movement that could be accounted for by treatment are unlikely to affect anxiety scores[31].

2.3.3 Open Field Test. Mice were placed in the center of an open 40*40*40cm chamber and allowed to freely explore for 30 minutes. Time in each zone (center or edges), entries into each zone, and distance traveled were automatically calculated using ANYmaze software. The ratio of time spent in the edges vs. center was calculated as a measure of anxious behavior, with higher values indicating higher anxiety-like behaviors. The center zone consisted of 25% of the total field (20*20cm). Total distance traveled was used to assess mobility[32].

2.4 Sub-chronic Stress Protocol

A subset of free-cycling female mice (*n*=72) were randomly assigned to a subchronic stress condition (n=36) or a no-stress control condition (n=36). In each condition, mice were randomly assigned to receive either the GPR171 agonist (MS15203), antagonist (MS21570), or vehicle (10% DMSO in saline). Mice in the stress condition underwent a six-day sub-chronic stress paradigm adapted from Baugher et al.[33]. The stress paradigm consisted of three different stressors presented to the mice in sequential order. Mice were exposed to the forced swim (Days 1 and 4), one hour of tail suspension (Days 2 and 5), and one hour of restraint stress (Days 3 and 6). The forced swim test was conducted on days 1 and 4 as described in section 2.3.1. In the tail suspension stressor, mice were suspended above the ground by their tails for one hour on days 2 and 5[34]. In the restraint stressor, mice were placed in a 50mL conical tube with

holes added, for one hour on days 3 and 6[33].

Fifteen minutes prior to each stressor, mice were administered their assigned drug. Mice in the no-stress condition were handled and injected with drugs each day, but were not exposed to the stressors. Following the last day of the paradigm, all mice were subjected to the Open Field Test (OFT), Elevated Plus Maze (EPM), and Forced Swim Test (FST), in the absence of any drug to avoid acute effects of the drug on animal behavior and to evaluate the effect of subchronic agonism and antagonism of GPR171 and the receptor's interaction with stress and anxious and depressive behaviors.

2.5 Ovariectomies

A subset of female mice (n=32) underwent aseptic surgery to remove their ovaries while under isoflurane anesthesia. Mice were administered meloxicam analgesia (5mg/kg) before surgery and 24hrs post-surgery. The surgical procedure involved creating a 5-10 mm incision in the lumbar region, followed by ligation of the Fallopian tubes and excision of the ovaries. Afterwards, the muscle and skin planes were sutured, and mice were transferred back to their home cages once recovering from anesthesia. To prevent infection, mice received a daily application of triple antibiotic ointment for three days following surgery.

After a two-week recovery period, mice received either β-estradiol supplementation (200ug/kg, s.c.; Cat. E8875 Sigma-Aldrich) or vehicle treatment (sesame oil; Cat. S3547 Sigma-Aldrich) once a day for four days. This treatment course was selected based on a previous study showing maximal antidepressant effects in the forced swim test of estradiol at the chosen dose and treatment course[35]. 15 minutes

following the last hormone injection, mice were administered either the GPR171 agonist or vehicle and subjected to the forced swim test 15 minutes later in order to assess GPR171 activity interaction with controlled estrogen levels.

2.6 RT-qPCR

RT-qPCR was performed as described in Ram et al.[13]. Immediately following the final day of behavioral testing, a random subset of subjects (4-5 animals per group) were euthanized by cervical dislocation and decapitation. Brains were removed, and the following areas were dissected and snap-frozen on dry ice: Prefrontal cortex (PFC), Nucleus Accumbens (NAc), Habenula (Hab), Basolateral Amygdala (BLA), and Ventral Hippocampus (vHipp). RNA was extracted from tissues using Trizol (Cat. 15596026, Invitrogen) and RNeasy Plus Mini Kit (Cat. 74136, Qiagen). RNA was quantified, and cDNA was synthesized using the Maxima first-strand synthesis kit for RT-qPCR (Cat. K1642, Thermo-Fisher). Samples were prepared using Universal SYBR Green Supermix (Cat. 1725121, Bio-Rad) and primers specific for GAPDH (housekeeping), ProSAAS, and GPR171 (IDT DNA Technologies). Gene expression analysis was done on a real-time thermocycler. The synthesized cDNA was assayed in triplicate. Results were analyzed using the $2^{-\Delta\Delta Ct}$ method in which $\Delta\Delta Ct = \{(Ct_{target\,:\,treatedsample} - Ct_{GAPDH\,:\,treatedsample}) - (Ct_{target\,:\,controlsample} - Ct_{GAPDH\,:\,controlsample})\}$. Negative control reactions were performed to ascertain contaminant-free cDNA synthesis, and primer specificity was evaluated using melt curve analyses.

2.7 Statistical analyses

Statistical analyses of behavioral data were generated by using one-way or two-way ANOVAs, when appropriate, using Prism Software (version 10.0, GraphPad Software). Post hoc tests were conducted to make pairwise comparisons, when appropriate. Statistical analyses of all RT-qPCR data were generated using t-tests. The ROUT method[36] was used to identify and exclude outliers. Results are presented as mean ± standard error of the mean (SEM).

3. Results

3.1 Acute GPR171 treatment decreases anxiety-like behaviors in female mice

In order to assess the effect of acute GPR171 agonism or antagonism on anxiety- and depressive-like behaviors, we exposed female mice to either the forced swim test, elevated plus maze, or open field test 15 minutes after injection of either the GPR171 agonist, antagonist, or vehicle. Agonist- and antagonist-treated females displayed no difference in immobility in the forced swim test compared to vehicle treated controls ($F(2, 33) = 0.16$, $p = 0.86$; Figure 1A). Similarly, GPR171 treatment had no effect on time spent in closed arms vs. open arms in the elevated plus maze ($F(2, 24) = 0.004$, $p = 0.99$; Figure 1B).

However, there was a main effect of treatment on time spent in the edges vs. the center of the open field test ($F(2, 24) = 3.067$, $p = 0.065$), with post hoc tests indicating that the GPR171 antagonist reduced anxiety-like scores by 53% when compared to the vehicle-treated mice (Dunnet's Multiple Comparisons $p < 0.05$; Figure 1C). There were no baseline differences in overall movement associated with the different treatment groups as measured by the total distance travelled in the open field test ($F(2, 25) = 0.832$,

p = 0.446; Figure 1D).

As behavior can vary slightly dependent on estrous cycle stage, we examined the estrous cycle phase of each mouse after the EPM and OFT via vaginal smears. There was no significant interaction between estrus stage and treatment results in either the open field test or elevated plus test ($F(4, 18) = 0.36$, $p=0.83$; $F(4, 19) = 0.487$, $p = 0.74$; respectively. Figure not shown). However, despite high samples size for treatment comparisons, estrous cycle comparisons were underpowered, which does not eliminate the possibility of estrogen interactions.

Figure 1

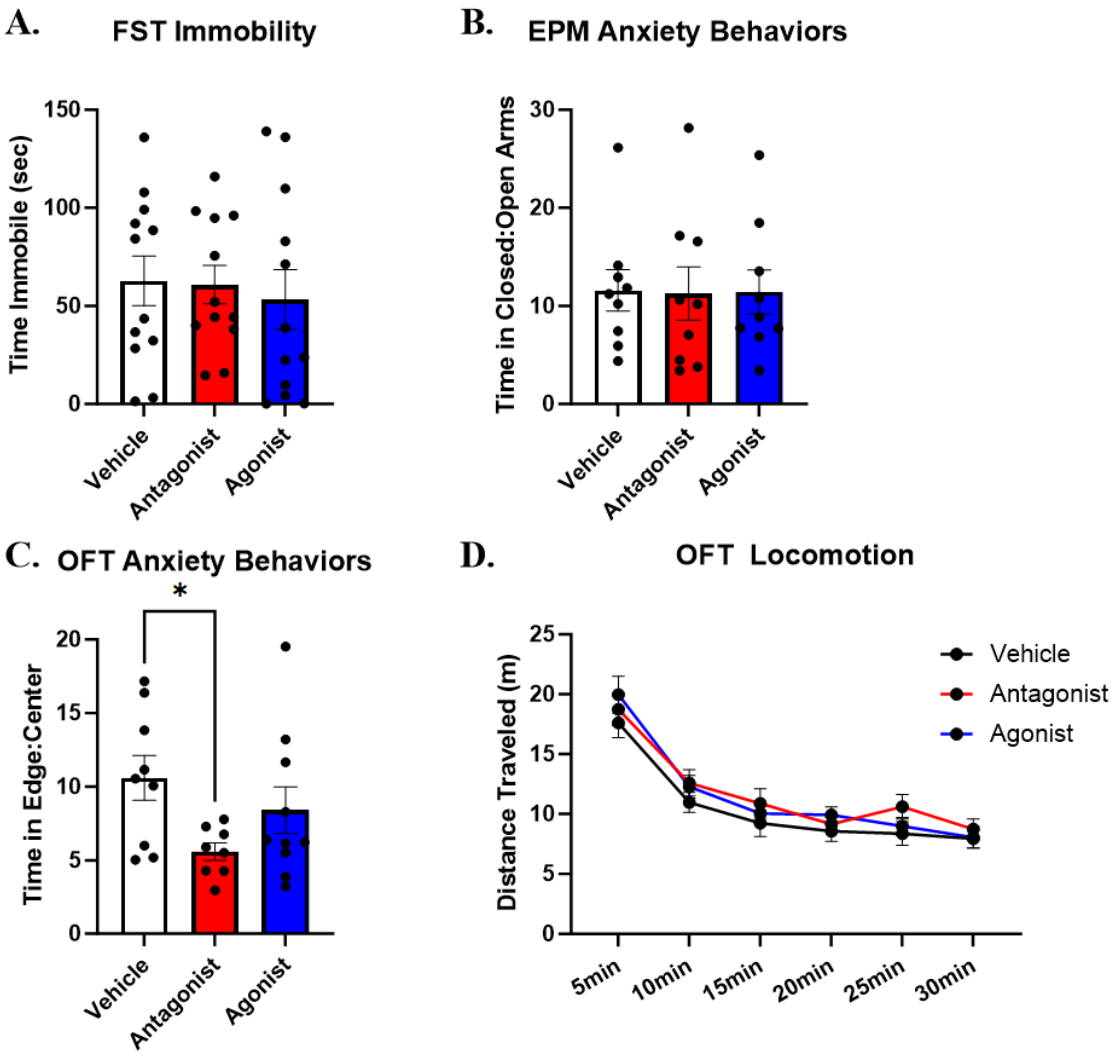

Figure 1. Systemic injection of GPR171 antagonist decreases anxiety-like behaviors, but not depression behaviors. (A) Systemic injection of GPR171 agonist, MS15203 (n=12) or GPR171 antagonist, MS21570 (n=12) did not alter depression-like behavior on the forced swim test compared to vehicle-treated mice (n=12). (B) Systemic injection of the antagonist (n=9) or agonist (n=10) did not result in any differences in time spent in closed vs open arms compared to vehicle-treated controls (n=10). (C) Systemic injection of the GPR171 antagonist (n=9) reduced time spent in the edges compared the center of the open field test indicating a decrease in anxiety-like behaviors when compared to the vehicle treated mice (n=9) and agonist-treated mice (n=10). (D) Systemic injection of antagonist (n=9) or agonist (n=10) did not alter locomotor activity over a period of 30 minutes in the open field test compared to vehicle-treated mice (n=9). A-C were analyzed with one-way ANOVAs. D was analyzed as a two-way ANOVA. * $p < 0.05$

3.2 Subchronic GPR171 Treatment Alters Anxiety Behaviors in a Stress-Dependent Manner

In order to assess the interaction between GPR171 signaling, stress, and anxiety/depressive-related behaviors, we exposed females to either 6 days of subchronic stress or no stress, while treated with the GPR171 antagonist, agonist, or vehicle before each daily stressor. Following the last day of stressors, we measured anxiety and depressive behaviors in the absence of any drugs.

In the elevated plus maze there was a significant interaction between stress condition and treatment ($F(2, 56) = 8.36$, $p < 0.001$). Post hoc analyses reveal that within the no-stress condition, repeated agonist treatment resulted in increased anxiety-like behaviors compared to the vehicle and antagonist treatment, as indicated by increased time spent in closed arms relative to open arms (Tukey's HSD, $p<0.001$ and $p<0.01$ respectively). This increase in anxiety-like behaviors was abolished in the mice that underwent subchronic stress (Tukey's HSD, $p > 0.05$). In the EPM, there was also a significant effect of drug treatment group ($F(2, 56) = 3.33$, $p<0.05$), with a trending main effect of stress condition ($F(1, 56) = 2.50$, $p=0.11$); Figure 2A).

In the open field test, there was a significant main effect of stress ($F(1,56) = 9.206$, $p<.001$) and treatment ($F(2, 56) = 4.392$, $p<0.05$, Figure 2B), with mice in the vehicle-treated stress group displaying higher anxiety-like behaviors relative to the no-stress vehicle-treated mice (Tukey's HSD, $p < 0.05$) and mice in the agonist-treated stress group displaying higher anxiety scores relative to the agonist treated controls (Tukey's HSD, $p < 0.001$). When evaluating the effect of stress and treatment on locomotion in the open field test, there was a main effect of stress, in which mice in the stress group

displayed hyperlocomotion ($F(1, 59) = 11.79$, $p<.001$; Figure 2C).

Depressive-like behaviors in the last four minutes of the forced swim test were not impacted by stress ($F(1, 66) = 0.138$, $p = 0.71$) or treatment ($F(2, 66) = 1.06$, $p = 0.35$, Figure 2D). However, analyses of all six minutes of the forced swim test revealed a strong effect of both time ($F(5, 66) = 6.847$, $p< 0.001$) and treatment ($F(4.4, 291) = 16.73$, $p<0.001$, Figure 2E), with post hoc analyses revealing increased immobility in all stressed groups compared to the no-stress groups in the first minute, with no differences by minute three.

Figure 2

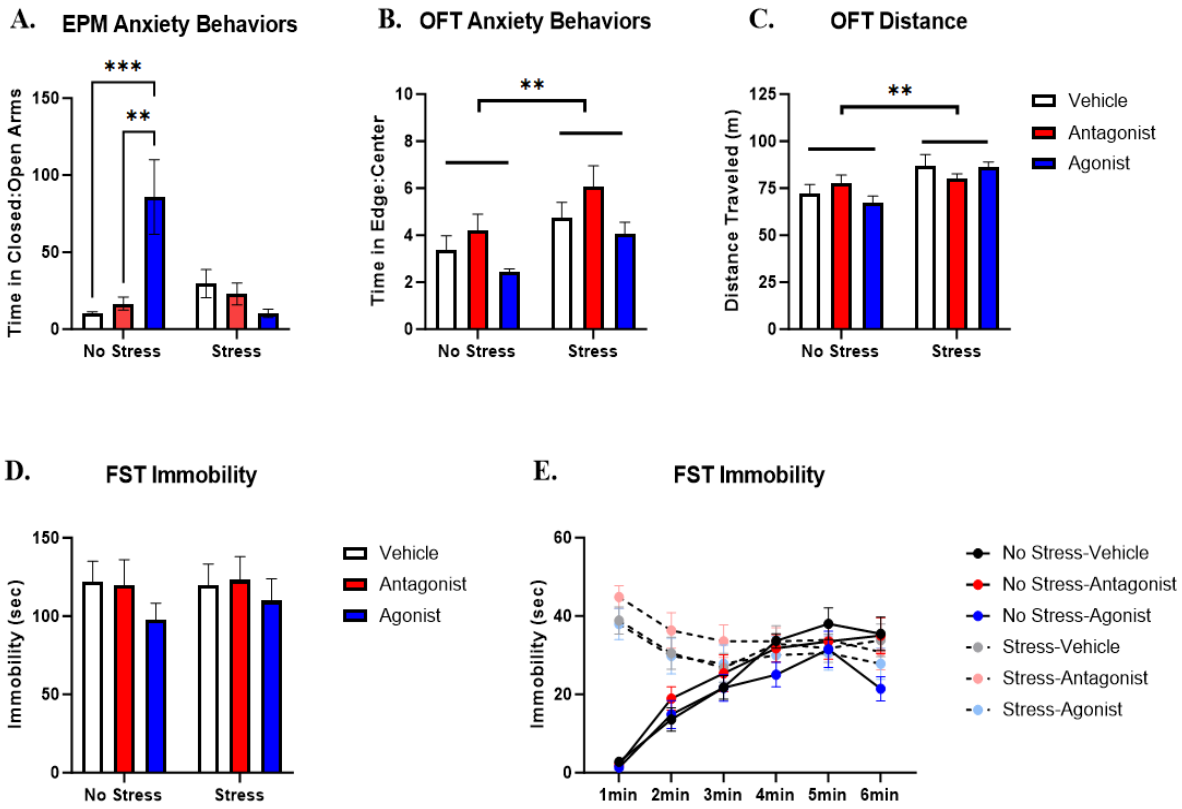

Figure 2. Subchronic injection of GPR171 agonist alters anxiety-like behaviors, in a stress-dependent manner. (A) Subchronic agonist injection increases anxiety-like behaviors in the elevated plus maze in animals that did not undergo the six subchronic stress paradigm, but not in animals that underwent the stress paradigm as compared to vehicle-treated mice (B) Mice subjected to the subchronic stress paradigm displayed increased anxiety-like behavior in the open field test, independent of GPR171 treatment when compared to mice in the no-stress group. Both vehicle treated and agonist treated mice displayed increased anxiety behaviors in the stress group relative to the no-stress mice (C) Mice in the stress groups displayed increased locomotion compared to mice in the no-stress groups. (D) Evaluation of the final four minutes of the forced swim test did not reveal any stress-dependent or treatment-dependent differences between groups. (E) Evaluation of all six minutes of the forced swim test revealed significant differences between the stress and no-stress groups only within the first two minutes. Sample size were as follows for all experiments: n=12 for each group. A-E were analyzed using two-way ANOVAs. *p <0.05, **p<0.01, ***p<0.001

3.3 Estrogen Levels Alter GPR171 Agonist Activity

To determine if estrogen modulates GPR171 drug actions, we performed ovariectomies on 32 female mice and supplemented each with either estradiol or placebo, followed by treatment with either the GPR171 agonist or vehicle. Mice were then tested on the forced swim test – a test that is sensitive to estrogen differences[35]. A two-way ANOVA displayed a significant interaction between estrogen treatment and GPR171 treatment ($F(1, 28) = 6.948$, $p<0.05$, Figure 3). In placebo-treated mice, GPR171 agonist resulted in slightly reduced immobility compared to the GPR171 vehicle (Tukey's HSD, $p=0.11$) indicating a decrease in depressive behaviors. In mice treated with estrogen, GPR171 agonist treatment increased depressive-like behaviors compared to the vehicle ($p<0.05$). There was also an effect of estrogen level overall ($F(1, 26) = 4.103$, $p = 0.05$) indicating that estrogen had antidepressant effects.

Figure 3

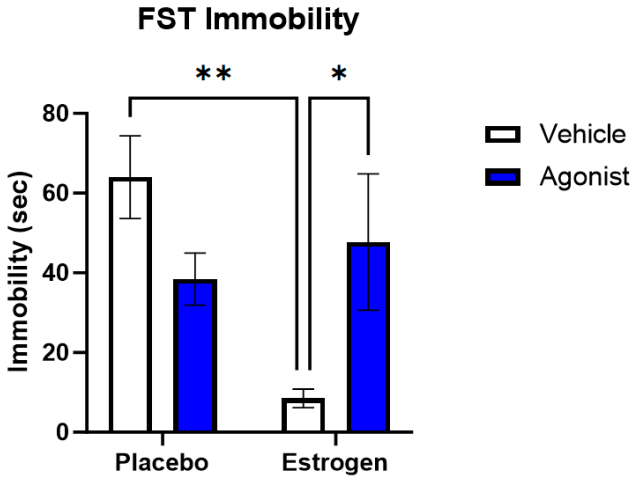

Figure 3. GPR171 agonist activity interacts with estrogen. After four daily injections of estrogen, mice that were injected with the GPR171 agonist displayed increased levels of immobility compared to those injected with the vehicle. After four daily injections of the placebo, mice injected with the GPR171 agonist displayed lower levels of immobility compared to those injected with the vehicle. The interaction between GPR171 agonist treatment and estrogen treatment is significant. Results were analyzed with a two-way ANOVA, *n*=8/group. *p<0.05, ** p< 0.01.

3.4 GPR171 Expression is Sensitive to Estrogen but not Subchronic Stress

Following Experiment 2 and 3, brains were dissected from vehicle-treated mice. A random subset of brains was used to explore the effects of stress vs. no stress (exp 2) and estrogen vs. placebo (exp 3) on GPR171 and ProSAAS expression through RT-qPCR analysis. Students t-tests did not reveal any changes in GPR171 expression or ProSAAS expression in mice that underwent stress compared to those who did not undergo stress in any of the brain regions analyzed (all $p>0.05$; Figure 4A and 4B).

Mice that were treated with estrogen for four consecutive days displayed approximately 40% decreased GPR171 expression in the Nucleus Accumbens and ventral hippocampus compared to placebo treated mice ($p<0.05$). Estrogen-treated mice also displayed a 47% increase in ProSAAS expression in the Nucleus accumbens ($p<0.001$). No other changes were evident in GPR171 expression or ProSAAS expression in the brain regions analyzed (Figure 4C and 4D).

Figure 4

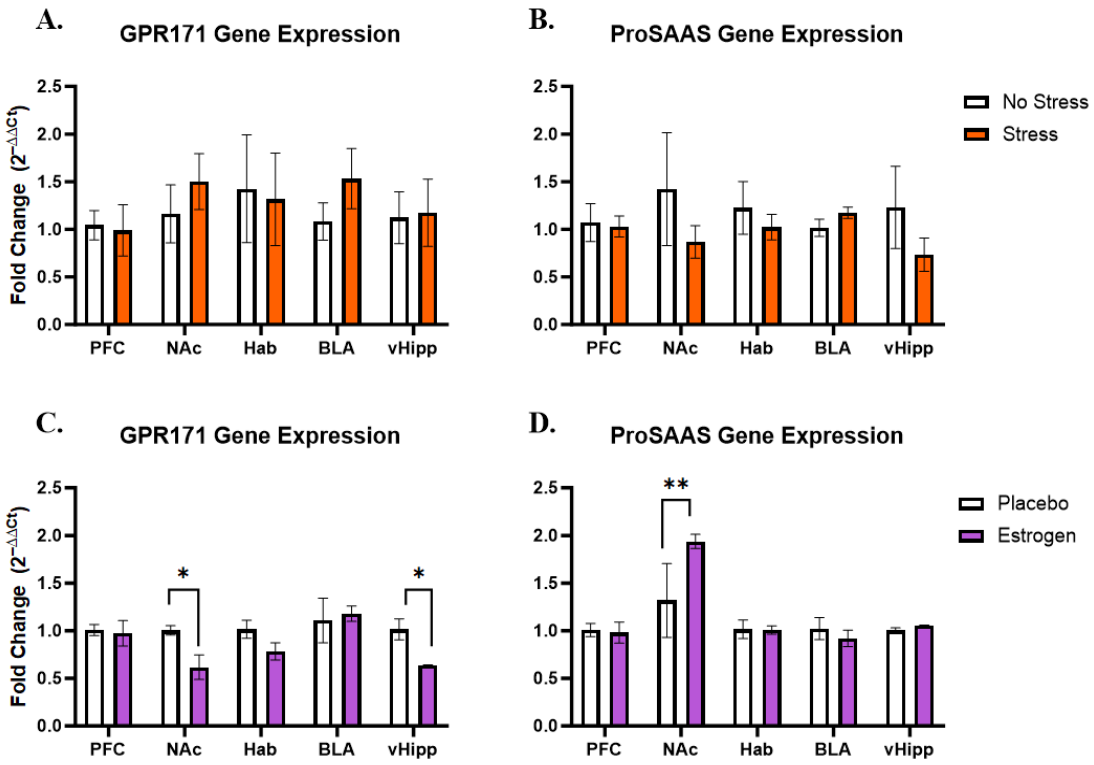

Figure 4. GPR171 and ProSAAS expression levels are altered by estrogen treatment but not stress. (A-B). Mice that underwent subchronic stress (*n*=5) did not display any changes in GPR171 (A) or ProSAAS gene expression (B) compared to mice that were not exposed to subchronic stress (*n*=5). (C) Mice that received four days of estrogen replacement (*n*=4) displayed decreased GPR171 expression in the Nucleus Accumbens and ventral hippocampus in comparison to placebo-treated mice (*n*=4). (D). Mice that received estrogen replacement displayed increased expression of ProSAAS in the Nucleus Accumbens relative to placebo-treated controls. Acronyms: PFC – prefrontal cortex, NAc – Nucleus Accumbens, Hab – Habenula, BLA – Basolateral Amygdala, vHipp – ventral hippocampus. All analyses consisted of Students t-tests. *$p<0.05$, **$p<0.01$

4. Discussion

In this study, we demonstrate that the GPR171 antagonist, MS21570, reduces anxiety behaviors in female mice. Additionally, we show that subchronic treatment of the GPR171 agonist, MS15203, results in heightened anxiety behaviors, which was abolished when mice were exposed to subchronic stress. This interaction between stress exposure and GPR171 treatment appears independent of any alterations in GPR171 or ProSAAS expression in key brain regions. Finally, we show that there is an interaction between GPR171 agonist treatment effects and estrogen levels, which appears to be related to estrogen-dependent changes in GPR171 and ProSAAS expression in the nucleus accumbens and ventral hippocampus.

This study is the first investigation into the role of GPR171 in anxiety regulation specifically in females. We show that acute GPR171 antagonist treatment effectively reduces anxiety-like behaviors in female mice in the open field test. Previous research has shown that acute GPR171 antagonism also reduces anxiety behaviors in male mice in the open field, but not the elevated plus maze[3]. Interestingly, mice lacking ProSAAS, and consequently its peptide product BigLEN that binds to GPR171, display increased anxiety behaviors[7]. While these results contradict our findings that decreased GPR171 signaling reduces anxiety, other ProSAAS products also regulate anxiety[37], indicating that GPR171's role in anxiety behaviors is balanced by other ProSAAS peptide products and associated receptors (i.e. PEN and GPR83 respectively).

We also demonstrate that repeated injections with the GPR171 agonist increased anxiety behaviors in the elevated plus maze. Previous research has indicated that while repeated GPR171 agonist treatment does not alter behavior in females, it is effective in

males within a pain paradigm[13]. However, our findings reveal that in female mice, repeated GPR171 agonist treatment in the absence of stress effectively induces anxiety responses, suggesting that the previously reported reduced efficacy in females may be context specific. Notably, treatment with the GPR171 agonist or antagonist did not affect behavior in mice subjected to subchronic stress.

Stress susceptibility is believed to be a major contributing factor to the higher prevalence of mood disorders among females compared to males[28,29], and alleviating stress by administering anxiolytic drugs prior to exposure to stressors has been shown to mitigate some effects of chronic stress[38] . Nevertheless, we were unable to achieve similar stress reduction with subchronic GPR171 antagonist treatment. While the lack of behavioral changes could be attributed to tolerance or withdrawal from the treatment on the test day, this explanation is unlikely, as tolerance typically only develops after longer treatment paradigms with common anxiolytics[39], and withdrawal is not a common occurrence with non-sedative anxiolytic drugs[40]. The lack of behavioral effects may also be attributed directly to the stress paradigm used, as stress has been shown to reduce the efficacy of several known anxiolytic compounds (as reviewed by Haller[41]). As such, future research should explore if acute GPR171 antagonist treatment given after stressors is effective in reducing anxiety behaviors, to determine if the effects of the antagonist are muted due to repeated injection or due directly to the effects of stress.

The absence of changes in GPR171 and ProSAAS expression after subchronic stress indicate that the subchronic stress paradigm was not sufficient to alter receptor or ligand levels although there was a behavioral interaction between GPR171 activity and stress. Previous research has shown that the BLA seems to directly regulate the anxiolytic

actions of GPR171, in which viral knockdown of the receptor leads to a similar anxiolytic effect as seen with acute GPR171 antagonism[3]. While GPR171 may regulate the excitability of the BLA[3], our study indicates that GPR171 expression is not altered after subchronic stress, indicating it is unlikely to be a driving force in anxiety behaviors under stress conditions.

Besides the basolateral amygdala (BLA), other brain regions known to regulate stress and anxiety include the prefrontal cortex (PFC), where similar short-term stress protocols such as the one we implemented in our study have been shown to alter the expression levels of LittleSAAS, a peptide derived from ProSAAS[18]. However, we did not observe changes in GPR171 and ProSAAS expression in the PFC or other regions investigated, despite their known sensitivity to stress[42]. This result could suggest that the specific stress paradigm used might not have been salient enough to alter expression levels, or that the roles of these areas might be influenced through different pathways or receptor systems. Notably, the hypothalamus, which is rich in GPR171 expression[2] and a critical component of the body's stress regulation via the hypothalamus-pituitary-adrenal axis[43], was not evaluated in this study. Future studies should examine this region, especially since PEN—a different ProSAAS-derived product—is upregulated in the hypothalamus following stress[44]. This could provide further insight into the mechanisms by which stress affects GPR171 and its related peptides.

Although GPR171 and ProSAAS expression levels were largely unaffected by stress, there was a region-specific effect of estrogen. We show that GPR171 expression is downregulated in both the NAc and vHipp in mice that underwent estrogen treatment relative to placebo-treated females. In addition, ProSAAS expression is upregulated in

estrogen-treated mice in the NAc. While it is well known that estrogen alters the expression of neuropeptides and non-estrogen receptors in the brain [16,17,45], this is the first study to show that GPR171 and ProSAAS are sensitive to estrogen. Of particular interest is the changes associated with the NAc. GPR171 agonist reduces excitatory transmission into the nucleus accumbens and alters dopamine signaling[9]. Dopamine signaling in the NAc is also directly influenced by estrogen[46]. While research has yet to show a functional interaction between GPR171 and estrogen in the NAc, it is likely that the two are working on similar circuits to control behavioral outputs. In addition, the changes seen in GPR171 expression in the vHipp are also critical to consider as the vHipp projects to the NAc and directly regulates susceptibility to depression[47].

The changes in receptor expression from estrogen treatment could explain, at least in part, the behavioral changes seen in the forced swim test between estrogen-treated and placebo-treated mice. Our results demonstrate a strong interaction between estrogen level and GPR171 agonist effects. Specifically, mice that underwent ovariectomies and subsequent estrogen replacement displayed increased depressive behaviors after receiving the GPR171 agonist compared to mice that received a vehicle. Meanwhile, mice that were not given hormone replacement displayed a decrease in depressive behaviors after receiving the GPR171 agonist, relative to their vehicle-treated counterparts. Previous research has shown that a reduction of estrogen levels in mice via ovariectomy decreases or completely eliminates the efficacy of common antidepressants, which is restored upon estrogen replacement[48]. This ability of estrogen to regulate the level of other neuropeptides and the actions of pharmaceuticals could explain why male mice, with significantly lower levels of estrogen[49,50], exhibit different responses to GPR171 agonism

than females[13,15].

Several limitations must be considered when interpreting the findings of our study. First, the two anxiety tests used, although part of the same analytical framework, represent different aspects of anxiety and do not necessarily always correlate with each other[51–53]. To capture the complexities of anxiety behaviors more accurately, additional tests such as the marble burying test, the zero maze, and social anxiety test should be considered. Furthermore, while our subchronic stress paradigm was effective in inducing increased anxiety behaviors, longer stress paradigms might provide a clearer understanding of GPR171's role in stress. Finally, while the supraphysiological estrogen treatment doses used in our study align with those used previously[48], a lowered physiological treatment could yield further insights into the interactions between GPR171 and estrogen.

Collectively, our findings demonstrate the potential of GPR171 as a promising target for anxiety treatment in females, while highlighting a potential interaction of GPR171 treatment activity with stress modulation. Moreover, our results highlight the significant influence of estrogen on receptor dynamics and subsequent behavioral outcomes. Notably, the differential responses to GPR171 modulation under varying stress levels and hormonal states underscore the importance of considering sex and hormonal status in the pharmacological management of anxiety in females.

CHAPTER III

GPR171 IS NECESSARY FOR NORMAL PHYSIOLOGICAL FUNCTIONS AND MOOD-RELATED BEHAVIORS IN MALES, BUT NOT FEMALES

1. Introduction

G protein-coupled receptors (GPCRs) are the largest and most versatile class of membrane proteins. Due to their substantial involvement in pathophysiology, and ease of manipulation, GPCRs are excellent pharmaceutical targets. Currently, approximately a third of all drugs approved by the FDA exert actions via GPCRs, and over 300 new GPCR drugs are under clinical trial[54]. While several GPCRs have clear, well-defined roles, other receptors, such as GPR171, are only beginning to be understood.

GPR171 is a recently de-orphanized G protein-coupled receptor (GPCR)[2] whose biological functions are still largely unknown. Thus far, studies have revealed a positive correlation between eating behaviors and GPR171 activity[2,3,6], anxiolytic effects of GPR171 antagonism[3], and improvement of neuropathic and inflammatory pain upon GPR171 agonism[13]. However, roles beyond those listed have not been studied extensively, and there is a lack of research examining sex differences in GPR171 functions. Given the therapeutic potential of GPR171, there is a pressing need to explore the roles of GPR171 and whether it is necessary for a broad range of functions in both males and females using global knock-out mice.

While significant advances in our understanding of GPR171's functions have occurred since its initial deorphanization, research has been conducted using pharmacological approaches following primarily systemic administration. While this

provides a general picture of GPR171 drug actions, the endogenous functions of GPR171 remain unknown. The synthetic agonist and antagonist while relatively selective for GPR171, are subject to unknown pharmacodynamic and pharmacokinetic regulation[3,6]. In addition, the drugs have unknown half-lives and lack the affinity of the endogenous ligand. The endogenous ligand, BigLEN, or BigLEN antibodies, have the greatest affinity, but are considered unstable when used *in vivo* and it's unclear how effective they are when injected intracerebrally[2]. Alternatively, viral knock-down studies, while valuable, are only capable of assessing GPR171 actions in a limited area of the brain and have resulted in a 60% decrease in receptor levels, rather than complete knockout[3]. Finally, while a great deal of research has been conducted on ProSAAS, assessing the levels of ProSAAS or knocking out the peptide is an indirect measure of what may be happening through GPR171 or possibly through one of the other peptides cleaved from ProSAAS[20].

In order to study GPR171 functions without the limitations of pharmacological methods, a new GPR171 knock-out mouse model, developed using CRISPR technology, was created. This genetic knockout will allow us to study both the known functions of GPR171 in a more controlled manner and explore any potential other effects that GPR171 may be mediating in both males and females. This study involves a detailed behavioral characterization of GPR171 global knock-out mice (Experiment 1), and an evaluation of the necessity of GPR171 for normal anxious and depressive behaviors with an exploration of how the knock-out effects neural activation patterns (Experiment 2).

2. Material and methods

2.1 Subjects

Subjects consisted of GPR171 knockout (KO) mice (females, n=22, males, n=22), their wild-type (WT) littermate controls (females, n=18, males, n=15), and their heterozygous (HET) littermates (females, n=19, males, n=22). Mice were originally produced by Cyagen Biosciences (C57BL/6JCya-*Gpr171*^{em1}/Cya) and then bred in a colony maintained on a C57BL/6J background. Knockout was confirmed via RT-qPCR using brain tissue and by PCR amplification from ear biopsy samples. Viability of knockout was confirmed via analysis of Mendelian Ratios.

All mice were approximately 6-10 weeks, weighing 15-28g at the start of each experiment. Mice were group housed (four to five per cage), in a temperature and humidity-controlled room on a 12:12 light cycle (lights on at 0700). Testing took place between 0700 and 1900. Food and water were available *ad libitum* except during testing. All behavioral testing was conducted by experimenters blind to the genotype. Equipment was cleaned with 70% ethanol after each test to eliminate any residual odors. Male and female mice were tested in separate cohorts to prevent any cross-sex olfactory or stress-induced confounds. All experiments were completed at the same time each day to control for temporal variables. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health and were approved by the Utah State University Institutional Care and Use Committee.

2.2. Phenotype Characterization

Behavior tests were divided into two batteries, with tests arranged from least

stressful to most stressful in order to minimize carryover effects. Each test was conducted on separate days. Prior to the start of each test battery, mice were habituation and handled for at least two days. The first battery of tests assessed basic physiological functions. Tests were performed in the following order: Ataxia, gross motor function, sensorimotor test, open field test, elevated plus maze test, and the forced swim test.

The second battery involved a second cohort of mice that were individually housed and involved the following assessments: feeding and drinking, the sucrose preference test, food deprivation, and feeding after food deprivation.

2.3 Assessment of Basic Physiological Functioning

2.3.1 Parallel Rod Test. The parallel rod test was conducted as previously described[55]. Briefly, mice were placed in an apparatus consisting of a floor with parallel metal rods, and a base plate, surrounded by clear acrylic walls. The number of foot slips from the rods to the base plate was measured, and ataxia was automatically calculated by ANYmaze as the ratio of total movement over foot slips.

2.3.2 Wire Hang Test. In order to assess motor function and muscle tone, mice were placed on a wire cage top that was inverted and hung upside down for up to five minutes[56]. Latency to fall into a clean, padded cage approximately 35cm below, was recorded. Mice underwent three trials each, or until the completion of a successful trial and the average trial time was calculated.

2.3.3 Adhesive Removal Test. The adhesive removal test was performed in order to assess any changes in sensorimotor abilities between mice[57]. Mice were placed in their home cage alone, and a small adhesive was placed on the snout of the mouse. The time in which it took the mouse to remove the adhesive was recorded, with a cut-off time of 60 seconds. A total of three trials were conducted with at least 15 minutes between trials. The average of all three trials was taken.

2.3.4 Measure of Locomotion. Locomotion was measured in the open field test, over a period of 30 minutes as described as part of the anxiety measures below (section 2.4.4).

2.3.5 Measures of Feeding Behaviors. Mice were individually housed and their food intake, water intake, and weights were recorded for three days. Following three days of ad libitum food, mice were fasted for 24 hours. After the fast, food was reintroduced and intake was measured at 1, 2, 4, 8, and 24 hours after initial presentation[6].

2.4 Assessment of Mood-Related Behaviors

2.4.1 Forced Swim Test. Mice were placed in a 4-liter glass cylinder with approximately 2500mL of water (24-26° C) for six minutes. Immobility was automatically calculated using ANYmaze software. Immobility during the final four minutes of each test was used as a measure of depressive behavior/behavioral despair[30].

2.4.2 Sucrose Preference Test. The sucrose preference test serves as a measure of anhedonia. Mice were individually housed for a period of 3 days with access to two separate drinking bottles with regular water in their home cage to acclimate to the presence of a second bottle. Bottle weights were taken each day to account for location preferences. After the acclimatation period, one bottle was filled with 1% sucrose solution. Water and sucrose intake was measured daily by weighing each bottle. Bottle locations were switched at the start of each day to reduce any location bias confounds. Sucrose preference was calculated as the volume of sucrose intake over the total volume of fluid intake averaged over three days[58].

2.4.3 Elevated Plus Maze. The elevated plus maze (EPM) consisted of a platform with four arms – two with walls, two open – elevated approximately 40cm off the ground. Mice were placed in the center of the apparatus and allowed to freely explore for 15 minutes. The distance traveled, number of entries into each arm, time spent in each arm, and the percent of entries into the open arms were automatically calculated using ANYmaze software. The ratio of time spent in the closed arms vs. open arms was calculated as a measure of anxious behavior, with higher values indicating higher anxiety-like behaviors. By analyzing anxiety as a ratio of time in closed vs open arms, any potential differences in movement that could be accounted for by genotype are unlikely to affect anxiety scores[31].

2.4.4 Open Field Test. Mice were placed in the center of an open 40*40*40cm chamber and allowed to freely explore for 30 minutes. Time in each zone (center or edges), entries

into each zone, and distance traveled were automatically calculated using ANYmaze software. The ratio of time spent in the edges vs. center was calculated as a measure of anxious behavior, with higher values indicating higher anxiety-like behaviors. The center zone consisted of 25% of the total field (20*20cm). Total distance traveled was used to assess mobility[32].

2.5 Immunohistochemistry (IHC)

IHC was performed as described previously[4]. Briefly, 90 minutes following the end of the forced swim test, mice (3-4/group) were deeply anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde. Brains were dissected and postfixed with paraformaldehyde and sectioned into 50um sections using a vibratome. Free-floating sections containing the Basolateral Amygdala (BLA) were incubated in 1% sodium borohydride (1% in PBS) to decrease autofluorescence followed by permeabilization with Triton-X 100, blocking with normal goat serum, and incubation overnight in primary antibody for c-Fos (Rabbit anti-cFos, Abcam #Ab190289; 1:300 dilution). The following day, slices were washed and incubated in the secondary antibody (Alexa 488 Goat anti-rabbit, Cat. #A11034; 1:200 dilution), with subsequent washes and mounting with Prolong Diamond on slides. Microscopy was performed on the Keyence, with two images of the BLA per animal included in analyses. cFos cell counts were automatically calculated using the Keyence Hybrid cell count (BZ-H3C, Keyence). Cell counts were averaged across the two images for each animal.

2.6 PCR

PCR was performed in order to genotype each mouse using standard procedures[59]. Ear punches were collected from each animal prior to weaning. DNA was isolated from each sample using NaOH and incubated at 98°C for 60 minutes. Following incubation, isolated DNA was added to a PCR mastermix including GPR171 specific primers and DreamTaq Green PCR Master Mix (Fisher, Cat K1081). Samples were run in a pre-designed PCR program, followed by electrophoresis in a 2% agarose gel cast with SYBR DNA gel stain (Fisher, Cat S33102), with a DNA ladder and negative control sample.

2.7 RT-qPCR

In order to validate that the GPR171 knockout mouse was not expressing any gene products, RT-qPCR was performed as described in Ram et al.[13]. Briefly, mice were euthanized by cervical dislocation and decapitation, and brains were removed, and homogenized. RNA was extracted from tissues using Trizol (Cat. 15596026, Invitrogen) and RNeasy Plus Mini Kit (Cat. 74136, Qiagen). RNA was quantified, and cDNA was synthesized using the Maxima first-strand synthesis kit for RT-qPCR (Cat. K1642, Thermo-Fisher). Samples were prepared using Universal SYBR Green Supermix (Cat. 1725121, Bio-Rad) and primers specific for GAPDH (housekeeping) and GPR171. Gene expression analysis was done on a real-time thermocycler. The synthesized cDNA was assayed in triplicate. Results were analyzed using the $2-\Delta\Delta Ct$ method in which $\Delta\Delta Ct = \{(Ct_{target\,:\,HET} - Ct_{GAPDH\,:\,HET}) - (Ct_{target\,:\,WT} - Ct_{GAPDH\,:\,WT})\}$. Negative control reactions were performed to ascertain contaminant-free cDNA synthesis and primer specificity was evaluated using melt curve analyses. Knockout was confirmed via the absence of any GPR171 expression.

3.4 Statistical analyses

Statistical analyses of behavioral data and IHC data were generated by using one-way or two-way ANOVA, when appropriate, using Prism Software (version 10.0, GraphPad Software). A Chi Square test was generated to calculate knockout viability using Prism Software. Additional post hoc tests were conducted to make pairwise comparisons, when appropriate. The ROUT method[36] was used to identify and exclude outliers. Results are presented as mean ± standard error of the mean (SEM).

3. Results

3.1 GPR171 Knockout Confirmation

PCR was used for GPR171 knockout identification at the DNA level according to the protocol designed by Cyagen Biosciences. As shown in Figure 1A, WT mice were identified by a single band at 671bp, KO mice were identified with a single band at 401bp, and heterozygous mice were identified by the presence of both bands. RT-qPCR was used to confirm the GPR171 mouse knockout. WT mice had an average 2^-(ΔΔCt) of 1.06, while Heterozygous mice displayed lower expression values with an average 2^-(ΔΔCt) of 0.64 relative to the WT mice. GPR171 was not expressed in KO mice (Figure 1B).

Figure 1

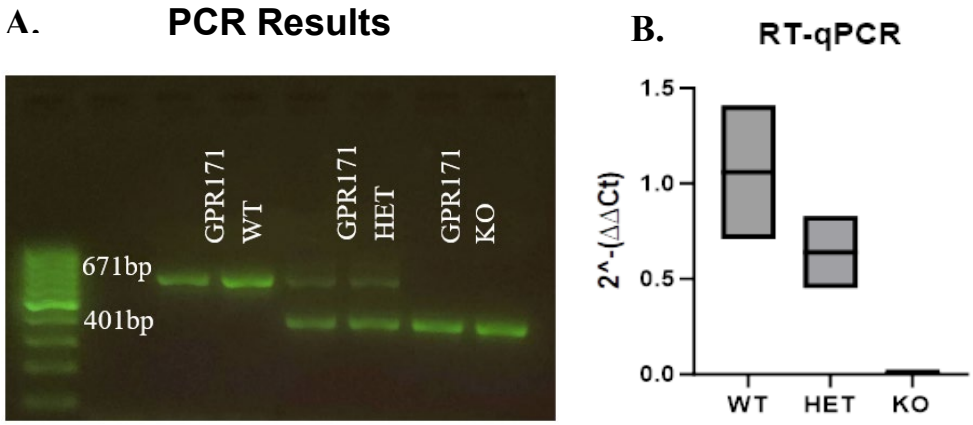

Figure 1. Confirmation of the GPR171 Knockout Mouse. (A). Mouse genotype was confirmed via PCR. KO mice display one band at 401bp, WT mice display one band at 671bp, and HET mice display both bands. (B). Confirmation of GPR171 gene expression was completed via RT-qPCR. HET mice displayed decreased GPR171 expression compared to WT mice, while KO mice did not display any gene expression.

In order to assess if the GPR171 knockout affected viability or fertility, a Mendelian ratio was calculated[60]. 151 mice from 5 different het*het breeding pairs were analyzed. A Chi Square test did not reveal any significant difference between groups (X^2 (1, N = 151) = 1.13, p = 0.25) indicating that genotype occurrence did not differ from expected ratios (Table 1 displays number of mice per genotype).

Table 1. Assessing for Knockout Viability

Genotype	Observed # of Mice	Frequency Observed	Frequency Expected
Wild-Type	48	31.79%	25%
Heterozygous	68	45.03%	50%
Knockout	35	23.18%	25%

3.2 GPR171 Knockout Affects Consumption Behaviors, Without Altering Weight in a Sex-Dependent Manner

In order to assess feeding and drinking behaviors, mice were individually housed for three days, and total food and water consumption was measured. There were significant differences in daily intake of food (Figure 2A) and water (Figure 2B) that mice consumed based on genotype ($F(2, 52) = 5.93$, $p < 0.01$; $F(2, 52) = 2.52$, $p = 0.09$ respectively). Post hoc analyses indicate that male GPR171 KO mice ate 20% more than their WT littermates (Tukey's HSD, $p < 0.01$ for both), while females did not differ by genotype (Tukey's HSD, $p > 0.05$). Male GPR171 KO mice also drank 30% less water than WT males (Tukey's HSD, $p < 0.05$), while females did not differ by genotype (Tukey's HSD, $p > 0.05$).

An analysis of the amount of food eaten after food deprivation revealed there were no significant differences in the amount of food consumed by genotype in either males ($F(2, 26) = 2.29$, $p = 0.12$) or females ($F(2, 26) = 0.49$, $p = 0.61$) when analyzed across all time points (Figure 2C). However, an analysis of feeding behaviors only after

the first hour of reintroducing food, revealed a significant interaction between genotype and sex ($F(2, 52) = 3.74$, $p<0.05$), with post hoc analyses indicating that in the GPR171 knockout mice, males ate 40% more food relative to females (Tukey's HSD, $p<0.01$; Figure 2F).

Finally, while KO mice on average weighed less than WT mice (a difference of 0.68g for males and 0.78g females), this genotypic difference was not statistically significant ($F(2, 52) = 0.49$, $p=0.61$; Figure 2E). These results were consistent when evening weights were taken after the mouse's inactive period (hours 0700-1900) ($F(2, 52) = 1.05$, $p=0.35$). Additionally, there were no significant differences in weight between genotype at any time point during the food deprivation period or immediately after in either males ($F(2, 26) = 0.20$, $p=0.81$) or females ($F(2, 26) = 0.20$, $p=0.81$) as seen in Figure 2D. While both males and females lost weight in response to deprivation, an analysis of the difference between starting weight before deprivation and weight 24 hours after food was returned revealed no genotype or sex specific differences ($F(2, 52) = 2.46$, $p=0.09$; $F(1, 52) = 0.07$, $p=0.77$, respectively).

Figure 2

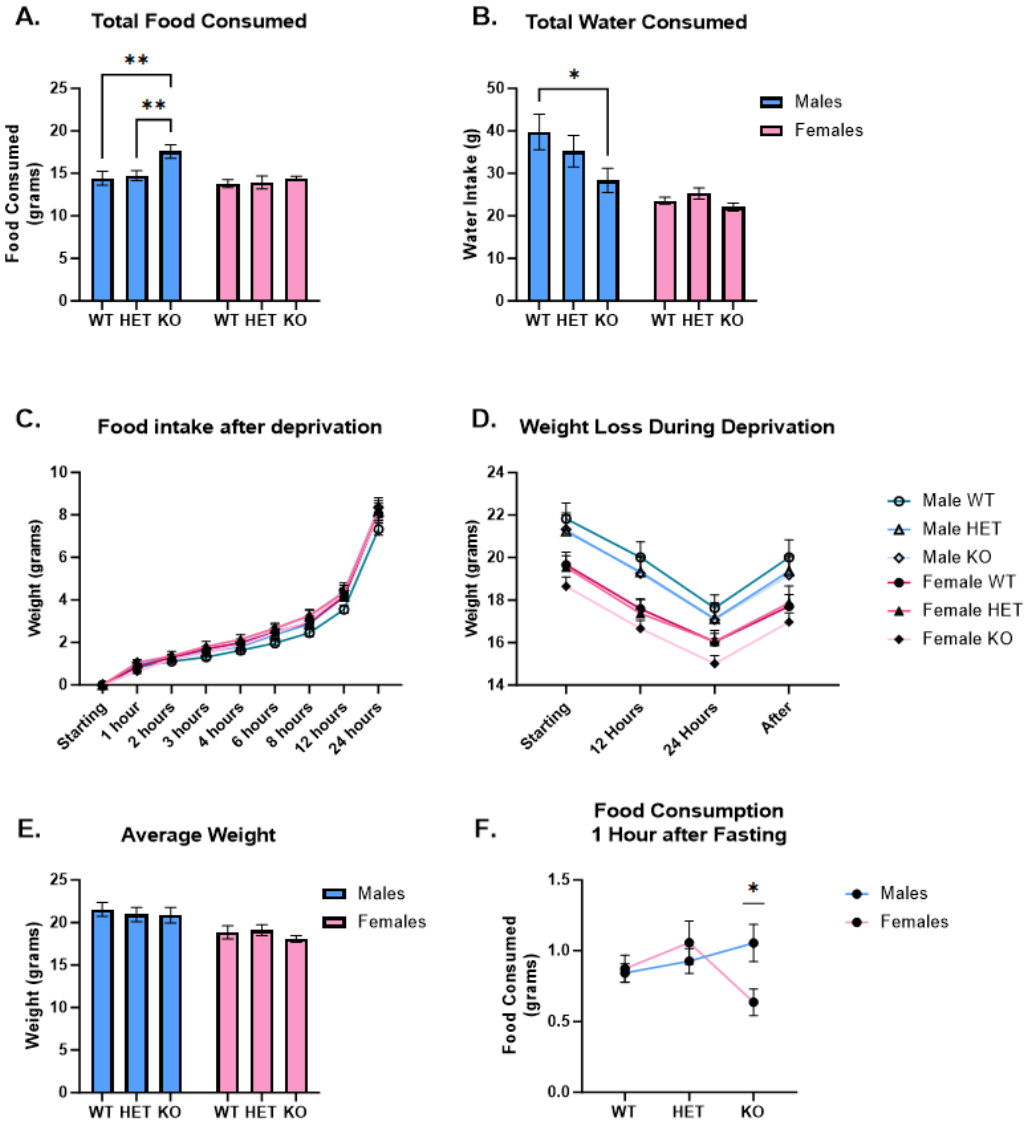

Figure 2. GPR171 Knockout Alters Consummatory Behaviors but not Weight in a Sex-Dependent Manner. (A). GPR171 male knockout mice ate significantly more food in a three-day period compared to WT males. Females were not affected by genotype. (B). KO male mice drank significantly less water than their WT littermates over a three-day period. Female mice were not affected by genotype. (C). Mice all ate food at approximately the same rate after a 24-hour fast when compared across all times and groups. (D). Mice all lost weight during a 24-hour fast, and regained it at approximately the same rate. (E). GPR171 knockout did not influence weight. (F). After the first hour of eating after a fast, male KO mice ate significantly more than female KO mice. All analyses were conducted as two-way ANOVAs. Sample sizes are as follows: Males (WT n=7, HET n=11, KO n =11) and Females (WT n=11, HET n =7, KO n=11). *p<0.05, **p<0.01

3.3 GPR171 is Not Necessary for Most Physiological Responses

GPR171 Knockout did not alter total distance traveled in the open field test ($F(2, 54) = 1.17$, $p=0.31$), and there were no differences in baseline locomotion between males and females ($F(1, 54) = 2.52$, $p=0.11$) as seen in Figure 3A.

In the wire hang test, the vast majority of mice reached the cut off trial time of five minutes (66%), indicating no gross motor abnormalities. Additionally, there was no significant difference between sex ($F(1, 54) = 0.18$, $p=0.66$) or genotype ($F(2, 54) = 1.29$, $p=0.28$) in time spent hanging (Figure 3B).

There was a significant difference in ataxia scores between males and females ($F(1, 51) = 5.05$, $p<0.05$), with males generally displaying greater ataxia than females. While genotype alone did not significantly alter ataxia ($F(2, 51) = 0.37$, $p = 0.69$), there was a moderate interaction between sex and genotype ($F(2, 51) = 2.52$, $p=0.08$) in which WT males and females did not differ significantly (Tukey's HSD, $p = 0.56$), but male HETs and KOs had ataxia scores that were 15% higher than females (Tukey's HSD, $p < 0.05$; Figure 3C).

In the adhesive removal test, while females took significantly longer to remove the adhesive compared to males ($F(1, 54) = 9.91$, $p < 0.01$), there was no significant main effect of genotype ($F(2, 54) = 0.31$, $p=0.72$), indicating that GPR171 genotype did not alter sensorimotor functioning (Figure 3D).

Figure 3

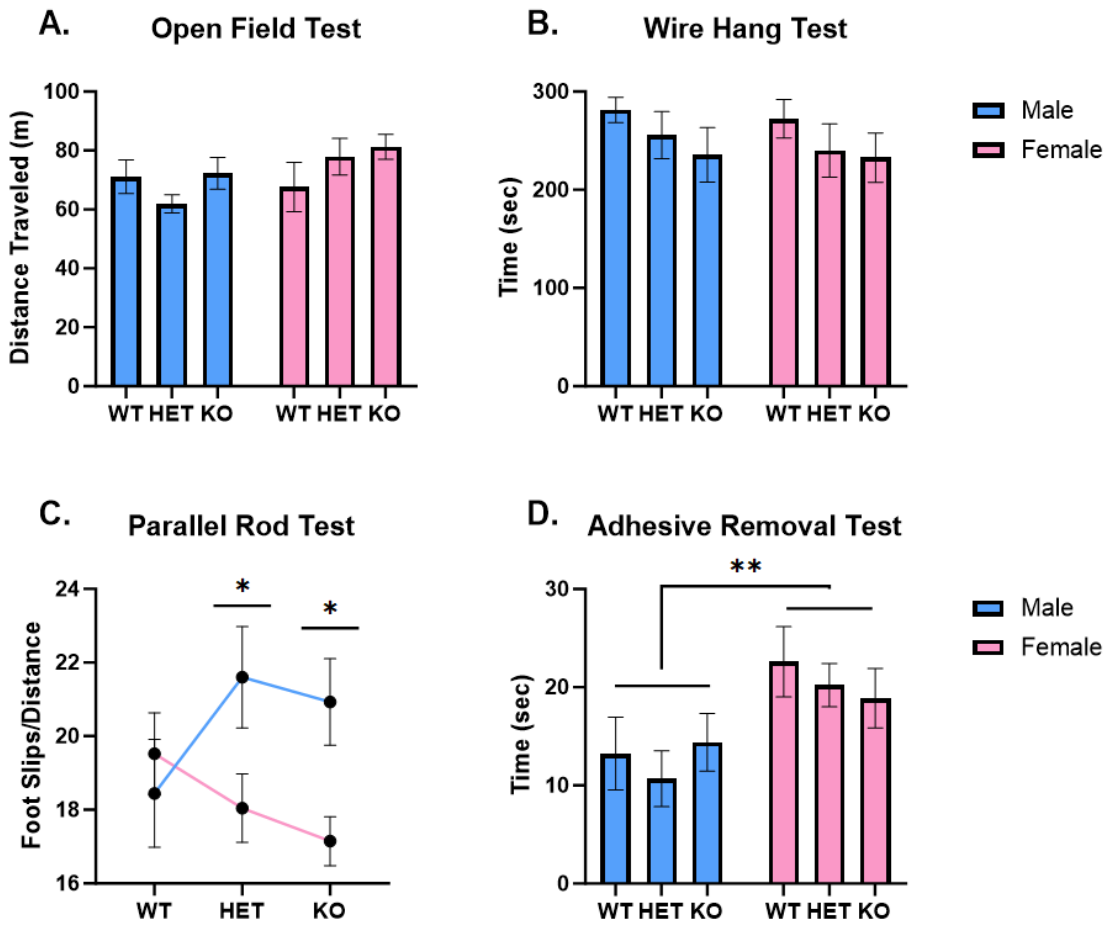

Figure 3. GPR171 is Not Necessary for Most Physiological Responses. (A). GPR171 knockout did not affect total distance traveled in the open field test. (B). GPR171 knockout did not alter the total time spent hanging in the wire hang test. (C). WT males and females did not differ in ataxia scores. Male HETs and KOs displayed higher levels of ataxia compared to female HETs and KOs. (D). There were no genotype specific differences in sensorimotor function, although males were significantly faster at removing an adhesive compared to females. All analyses were conducted as two-way ANOVAs. Sample sizes are as follows: Males (WT n=7, HET n=11, KO n =11) and Females (WT n=11, HET n =7, KO n=11). *p<0.05, **p<0.01

3.4 GPR171 Knockout Reduces Anxiety Behaviors in a Sex-Dependent Manner

In the open field test, male mice exhibited significantly lower anxiety scores compared to female mice as measured by the time spent in the center over the edges ($F(1, 49) = 5.06$, $p<0.05$). While there was no main effect of genotype on anxiety behaviors ($F(2, 49) = 0.20$, $p=0.81$), post hoc tests reveal that only WT mice differed in anxiety scores between males and females (Tukey's HSD, $p < 0.001$), while KO and HET mice did not differ by sex (Tukey's HSD, $p > 0.05$; Figure 4A).

In the elevated plus maze, there was a significant main effect of sex ($F(1, 48) = 4.47$, $p < 0.05$) and a main effect of genotype ($F(2, 48) = 3.14$, $p=0.05$). Post hoc analyses show that male HET and KO mice displayed less anxiety behaviors relative to male WT mice (Tukey's HSD, $p<0.05$, and $p=0.05$, respectively). However, there were no genotype specific differences in female mice (Tukey's HSD, $p>0.05$; Figure 4B).

Figure 4

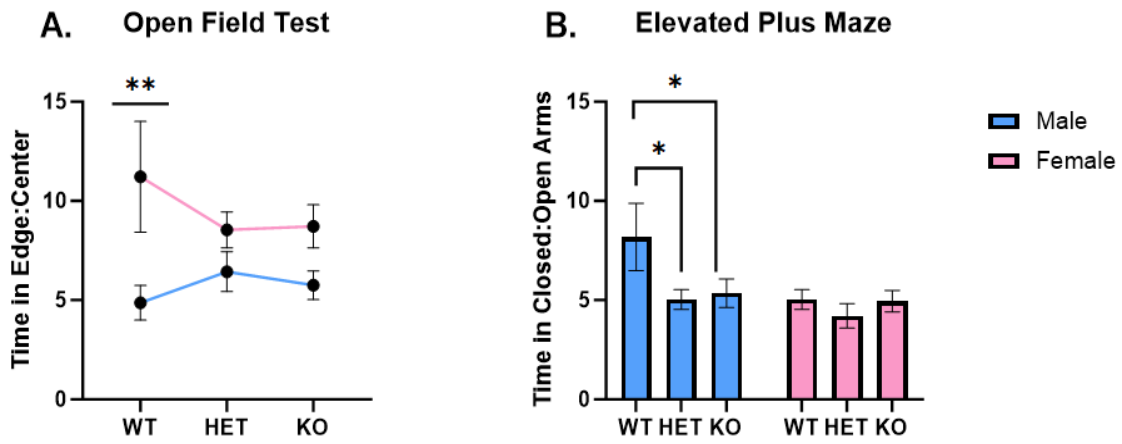

Figure 4. GPR171 knockout alters anxiety behaviors in a sex and test dependent manner. (A). In the open field test, WT females displayed increased anxiety behaviors relative to WT males. This sex difference was abolished in HET and KO mice. (B). In the elevated plus maze, GPR171 KO and HET mice displayed reduced anxiety behaviors relative to WT mice in males. Females were not affected by genotype. All analyses were conducted as two-way ANOVAs. Sample sizes are as follows: Males (WT n=8, HET n=11, KO n =11) and Females (WT n=7, HET n =12, KO n=11). *p<0.05, **p<0.01.

3.5 GPR171 Knockout Alters Depressive Behaviors and Decreases cFos Expression in the Basolateral Amygdala

In the sucrose preference test, there was a significant main effect of genotype on anhedonia behaviors ($F(2, 52) = 9.71$, $p < 0.001$), in which male KOs displayed significantly lower preference for sucrose water compared to WT and HET mice (Tukey's HSD, $p < 0.001$; Figure 5A). There were no significant main effects of genotype on total water volume consumed during the test ($F(1, 52) = 3.76$, $p = 0.06$). Interestingly, the genotypic effects on anhedonia behaviors were only present when calculated for consumption during the day time (0700-1900). Analysis of sucrose preference during the

night time (1900-700) did not reveal any significant genotype differences ($F(1, 52) = 1.92$, $p=0.17$; Figure 5B).

In the forced swim test, there was no main effect of either genotype ($F(2, 52) = 0.031$, $p = 0.96$) or sex ($F(1, 52) = 0.45$, $p=0.50$), however there was a trending interaction between the two variables ($F(2, 52) = 2.59$, $p=0.08$) indicating that genotype specific changes in immobility were dependent on sex. Specifically, while WT and HETs were not significantly different from each other between males and females (Tukey's HSD, $p > 0.05$), male KOs spent approximately 30% more time immobile compared to female KO mice (Tukey's HSD, $p < 0.05$; Figure 5C).

In order to determine if the behavioral differences were reflected by neuronal activation, cFos immunohistochemistry was performed on WT and KO mice of both sexes (see Figure 6 for representative images). Analysis was focused on the BLA. There was no main effect of sex on cFos activation ($F(1, 12) = 1.81$, $p=0.20$), but there was a trending main effect of genotype ($F(1, 12) = 3.46$, $p=0.08$) with KO mice displaying decreased activation compared to WT mice. Unlike the behavioral data, there was no interaction between genotype and sex ($F(1, 12) = 0.06$, $p=0.80$; Figure 5D).

Figure 5

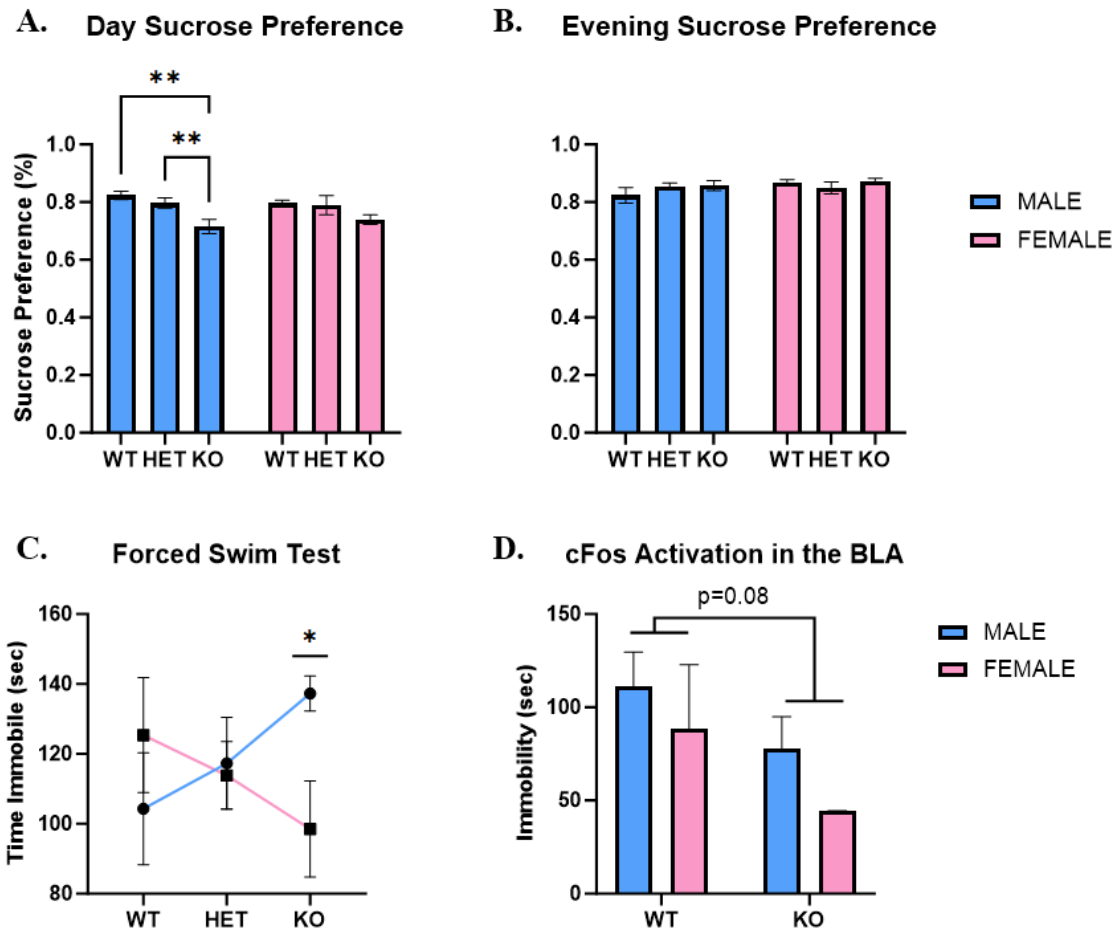

Figure 5. GPR171 knockout alters depressive-like behaviors in a sex and genotype specific way. (A). GPR171 knockout males displayed decreased sucrose preference relative to WT males when sucrose intake was measured during the day time (inactive period). (B). Sucrose preference during the night (mouse's active period) did not differ by sex or genotype. (C) In the forced swim test, there was a trending interaction between sex and genotype, with male KOs displaying higher immobility relative to female KO mice. (D). KO mice had lower cFos activation in the BLA relative to WT mice during the forced swim test. All analyses were conducted as two-way ANOVAs. Sample sizes are as follows for A-C: Males (WT n=8, HET n=11, KO n =11) and Females (WT n=7, HET n =12, KO n=11). Sample sizes for D are as follows: Males (WT n=5, KO n =5) and Females (WT n=3, KO n=4). *p<0.05, **p<0.01.

Figure 6

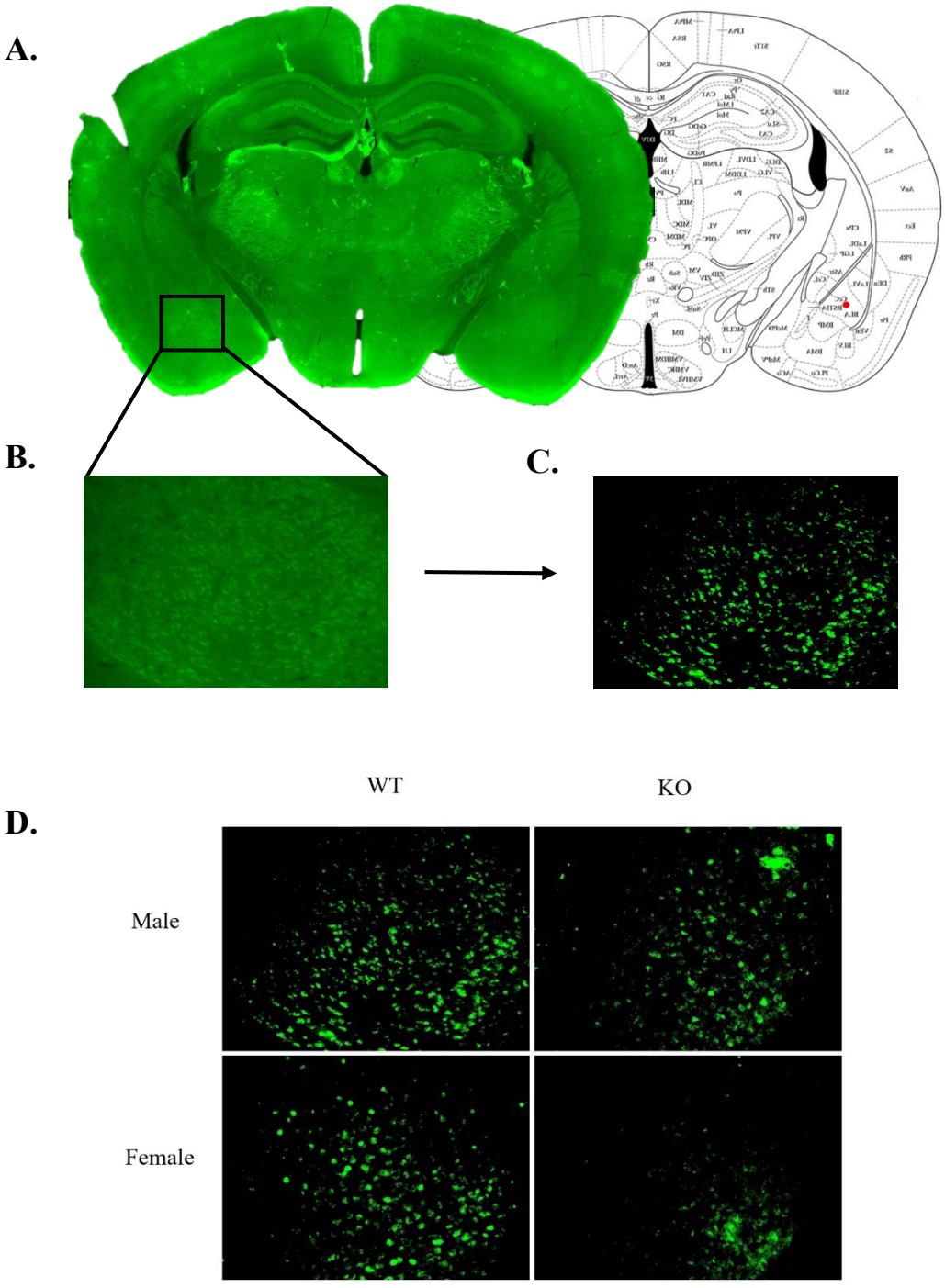

Figure 6. cFos Staining in the BLA differed by genotype. (A) An image taken on the Keyence with the BLA outlined by a black box (left). The associated image from the mouse atlas is included (right) with a red dot on the BLA. (B). An 20x image of the BLA with cFos staining. (C). The same image as in (B) edited to highlight only cFos positive cells. (D). Representative images of cFOS activity in the BLA of male and female WT and KO mice.

4. **Discussion**

In this study, we validated a new GPR171 knockout mouse model and explored the phenotypic patterns associated with the genetic deletion of GPR171. We show that male KO mice ate more and drank less, while female consummatory patterns were largely unaffected. Physiologically, GPR171 KO mice were similar in behaviors compared to WT mice, except in measures of ataxia. Additionally, male KO mice displayed less anxiety behaviors, and the GPR171 KO reduced the sex differences found in WT mice in the anxiety tests. Finally, depression and anhedonia tests show sex-specific alterations in behavior, while analysis of cFos activation during the forced swim test in the BLA revealed small genotypic changes.

The regulation of hunger and thirst is so vital to survival that it is estimated that over 20 different hormones, peptides, and receptors are involved in its maintenance[61,62], specifically in the hypothalamus. Our research shows that GPR171 is a pivotal component of these processes. Specifically, we show that the knockout of GPR171 led to increased food consumption and decreased water consumption in male mice. This supports previous research which has shown that viral knockdown of GPR171 in the hypothalamus increased food intake[2]. However, research using pharmacology has shown the opposite interaction in which increased GPR171 activity via acute administration of the GPR171 agonist, MS15203, or BigLEN, resulted in increased food intake, especially after a period of fasting[6,8]. Additionally, blocking GPR171 with the BigLEN antibody reduced feeding[2,63], and GPR171 antagonist injection stopped increases in food intake caused by AgRP neuron activation[3]. While research collectively indicates that GPR171 is highly involved in food intake, the direction of the effect differs based on the type of

manipulation. Of note, BigLEN is localized in AgRP/NPY neurons[63] – the most potent orexigenic peptides in the hypothalamus[64,65]. Our results indicate that GPR171, a $G_{i/o}$ receptor, likely maintains an overall tonic inhibition on the AgRP/NPY neurons, a response typical of $G_{i/o}$ receptors[66] and when that inhibition is removed via genetic knockout, food intake increases as does the motivation to eat after fasting. Meanwhile, short-term effects of GPR171 agonist or antagonist treatment likely exert much smaller effects on the hypothalamus, and regulate immediate food intake through different inputs into the hypothalamus, including a circuit from the nucleus accumbens to the lateral hypothalamus, which has been shown to regulate unrewarded persistence towards food, that is directly regulated by GPR171[9]. However, the overall effect of GPR171 knockout on neural circuitry involved in feeding requires investigation beyond this paper.

This is the first study, to the best of our knowledge, that explores the roles of GPR171 in basic physiological processes outside of feeding and drinking. We show that while GPR171 knockout does not alter basic locomotion, gross motor function, or fine-tuned sensorimotor function, KO male mice displayed increased levels of ataxia, relative to females. Ataxia – muscle incoordination - is generally associated with changes to the cerebellum, or its inputs including the frontal lobes, inner ear, posterior column of the spinal cord, and dorsal root ganglia[67]. Interestingly, GPR171 does not appear to be highly localized in areas of the cerebellum or brainstem specifically implicated in the development of ataxia including the vermis[68], vestibular nuclei[69], or vestibulocerebellum[70] (according to analysis of data from the Allen Brain Gene Expression Atlas[71]). However, GPR171 has been found to be located in the dorsal root ganglia[8] where it is proposed to modulate nociceptive functions. GPR171 in this area may

also contribute to ataxia. In addition, when ProSAAS is knocked out of mice, they displayed deficits in the visual placing test, in which KO mice needed to be far closer to a surface in order to register it visually[7]. Vision was not evaluated in this study, but should be evaluated in future studies as it plays a large role in motor coordination and ataxia[72]. In sum, the functional significance of ataxia in GPR171 KOs remains to be explored, with future studies including gait analyses and visual acuity tests, to validate the results observed in this study.

Beyond physiological roles, we show that GPR171 KO affects mood-related behaviors. The role of GPR171 in anxiety and depression has previously been established. GPR171 antagonist treatment reduces anxiety behaviors in males[3] and females (as discussed in Chapter 2). Our data corroborates these findings as decreased GPR171 signaling in GPR171 HET mice, and GPR171 deletion in GPR171 KO mice, reduces anxiety-behaviors in the elevated plus maze. However, we only observed these effects in males, as females displayed no genotype specific changes. Additionally, we observed similar sex-specific changes in depression with male KO mice displaying decreased sucrose preference during the day, while females were unaffected. Previous research indicates that GPR171 knockdown mice display increased activity during the night and increased water consumption compared to control mice. These differences were not apparent during the day time[2]. As such, it is possible that the differences we observe in the sucrose preference test in KO mice are reflective of general activity differences during the day and night relative to WT mice. The differences could also be related to motivation differences between genotypes, as GPR171 regulates motivation for food in the nucleus accumbens[9]. However, given that male mice also display increased

depressive-like behaviors in the forced swim test, it is possible that the sucrose preference test is a true reflection of anhedonia.

In conjunction with the forced swim test behavior, we examined the contribution of the BLA using cFos Immunohistochemistry. cFos is an immediate early gene that is transcribed with changes in afferent inputs or external stimuli and serves as a marker of neuronal activation[73]. cFos activation in the BLA is positively correlated with immobility time in the forced swim test[74], and BLA neuronal activity was greatly reduced in mice who remained consistently immobile in the forced swim test[75]. We show a similar pattern with female KO mice, that show both reduced immobility and reduced cFos expression in the BLA. However, male mice show a reduction in BLA cFos, but an increase in immobility. GPR171 is found on both GABA and glutamatergic neurons in the BLA of male mice[3] likely balancing the excitatory/inhibitory balance outputs of the area. This balance is sex-dependent as females display increased excitability of neurons in the BLA compared to males[76]. Despite these known sex differences, it is not clear why male and female knockouts show reduced cFos expression, yet have opposite behavioral outputs, although it may be possible that different populations neurons are activated between sexes. Additionally, while the BLA was the only area considered for this study, other areas must be considered in order to determine if the different neural circuits reflect the behavioral differences we see, including the vHipp, PFC, and NAc which are areas that regulate depressive behaviors in a sex-dependent manner[42].

In almost every test in which differences were found in our study, there were significant interactions between genotype and sex that must be taken into consideration. For example, in the elevated plus maze, WT males and females displayed differences in

anxiety-related behaviors, but HET and KO males and females did not differ, indicating that the genetic decrease and deletion of GPR171 reduced sex differences in anxiety behaviors. Sex differences are well established in the elevated plus maze, with females typically displaying lower anxiety related behaviors[77–79], while sex differences in the open field are not always typical[80] as we reflect in our data. This sex difference has been abolished by a variety of mechanisms including hormonal influences in which males with early gonadectomy displayed anxiety levels similar to females[81]. While no research has explored the interaction of male hormones and GPR171, we demonstrate in Chapter 2 that GPR171 interacts with estrogen and may have a functional relationship with the hormone. As such, GPR171 knockout may affect hormonal levels, and by association, sex differences noted in the tests. Alternatively, GPR171 knockout may affect specific circuits that are primarily involved in the male stress response, and not the female stress response, or vice versa[42]. Other peptide receptors such as oxytocin in the PFC can mediate anxiety-behaviors in males, but not females, representing an example circuit in which any genetic alteration could reduce known sex differences[82]. Other sex-specific circuits exist and are well documented, but it is not clear yet whether the observed decrease in sex differences in anxiety measures upon GPR171 knockout are due to specific circuits[42] or more global changes such as hormonal interactions.

With the creation of the new GPR171 knockout mouse model, we were able to explore the role of GPR171 in a wide array of behaviors that hadn't all been explored previously and clarify the necessity of GPR171 for several behaviors. While this study does not explore a comprehensive list of all behavioral paradigms, it provides the baseline for future studies to expand upon. In addition, we did not control for estrogen

interactions in our study, but the appearance of numerous interactions between sex and genotype warrants further research into this topic. Finally, in our study we did not explore results of GPR171 knockout in older mice, and it is well known that phenotypic changes can appear over time or further develop[83,84]. While we see very clear genotype differences at the age range in which we explored, it would be interesting to determine how these changes adjust over a broader time scale.

In conclusion, we show that GPR171 is necessary for normal consummatory behaviors, physiological functions, anxiety, and depression. Interestingly, all tests seemed to be regulated in a sex-dependent manner, with females largely unaffected in any behavioral paradigms by genotype. Together, this study sets the framework for further development of GPR171-targeted pharmaceuticals but with extra precaution to include both males and females in each study. Additionally, this GPR171 knockout mouse may prove a valuable research tool for cancer research as several studies have implicated a role for GPR171 in antitumor immunity[85], proliferation of cancer cells[86], and as a biological marker for cancer[87–89]. Collectively, this study provides the groundwork for the genetic dissection of GPR171 in multiple behaviors, while exploring a potential mechanism that mediates known sex differences in disorders and diseases.

CHAPTER IV

GPR171 KNOCKOUT REDUCES MORPHINE ANTINOCICEPTION WITHOUT AFFECTING BASELINE THERMAL AND MECHANICAL SENSITIVITY

1. Introduction

Pain affects millions of individuals, costing an estimated $560-$635 billion annually in the United States[90]. Acute pain is the most common reason for emergency department visits, with nearly 2/3 of emergency department patients treated for pain-related conditions[91]. Opioids, such as morphine, have long been considered the gold standard in analgesic drugs[92], but due to the dangerous addictive profile which has played a massive role in the opioid pandemic[93], new pain therapeutics are necessary. While a great deal is known concerning the neurobiology of pain, including the actions of nociceptors involved in the interpretation of stimuli (as reviewed in[94]) and the role of downstream modulation of pain signals from the periaqueductal gray (PAG)[95], new targets are necessary to develop better pharmaceutical treatments.

GPR171, a recently de-orphanized G protein coupled receptor, has been proposed as a potential new target for the management of pain. GPR171 agonism reduces neuropathic pain and inflammatory pain in male mice, but not females, at least in part due to actions in the PAG[13]. In addition, the discovery of GPR171 in nociceptors indicates that GPR171 may mediate not only the reduction of pain through central mechanisms, but also through peripheral mechanisms, and may play a critical role in the initial evaluation of painful stimuli[8].

Interestingly, morphine produces greater antinociception in both male and female mice when used in combination with a GPR171 agonist, compared to when either is used alone[4,15]. Furthermore, GPR171 antagonism reduces the antinociceptive effect of morphine[4], indicating that GPR171 might play a vital role in morphine antinociception. While the effects of GPR171 ligands are not due to binding to the orthosteric binding site of opioid receptors[4], the synergistic interaction between GPR171 ligands and morphine may be mediated via allosteric modulation of opioid receptors or through receptor heterodimerization in which GPR171 and mu-opioid receptors function together as a single unit[96]. Although GPR171 may prove a promising target for modulating acute pain in combination with morphine[4], it is unclear whether GPR171 is necessary for normal pain responses, and whether the synergistic effects of GPR171 agonist and morphine[4] are modulated via GPR171 or off-site drug targets.

Here we explored whether GPR171 is necessary for morphine antinociception, while also exploring if genetic knockout of GPR171 alters baseline thermal and mechanical sensitivity. To better understand GPR171's necessity in normal pain responses, we carried out three experiments: First, using male and female knockout (KO), heterozygous (HET), and wild-type (WT) mice, we assessed for any differences in thermal and mechanical sensitivity. Next, we injected a separate set of mice (males and females, WT, HETs, and KOs) with either the GPR171 agonist, morphine, a combination of the agonist with morphine, or a vehicle to assess for sex- and genotype-specific changes in antinociception on the hot plate and tail flick tests. Lastly, we analyzed the expression of multiple pain-related receptors and peptides in the PAG in naïve animals of both sexes and all three genotypes to determine any neuroanatomical changes associated

with GPR171 knockout. Taken together, this study sought to determine the necessity for GPR171 in pain and touch.

2. Material and methods

2.1 Subjects

Subjects consisted of GPR171 knockout (KO) mice (females, n=17, males, n=17), their wild-type (WT) littermate controls (females, n=21, males, n=16), and their heterozygous (HET) littermates (females, n=19, males, n=22). Mice were originally produced by Cyagen Biosciences (as described in Chapter 3) and then bred in a colony maintained on a C57BL/6J background. Genotypes were confirmed by PCR amplification from ear biopsy samples.

All mice were aged 7-12 weeks, and weighed 17-28g at the start of each experiment. Mice were group housed (four to five per cage), in a temperature and humidity-controlled room on a 12:12 light cycle (lights on at 0700). Testing took place between 0700 and 1900. Food and water were available ad libitum except during testing. For females, estrus cycle stage was confirmed via vaginal smear only on the last day of each experiment, as repeated vaginal smears cause stress in mice[97]. All behavioral testing was conducted by experimenters blind to the genotype and treatment group. Equipment was cleaned with 70% ethanol after each test to eliminate any residual odors. Male and female mice were tested in separate cohorts to prevent any cross-sex olfactory or stress-induced confounds. All experiments were completed at the same time each day to further control for temporal variables. All procedures were performed in accordance with the

Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health and were approved by the Utah State University Institutional Care and Use Committee.

2.2 Drugs

The GPR171 agonist, MS15203 (10 mg/kg, s.c.; as described in Afrose et al.[15]) and Morphine Sulfate (5mg/kg, s.c.; Santa Cruz Biotechnology, Inc., Santa Cruz, Ca.) were diluted in saline (Cat. 67-68-5 Sigma-Aldrich). All drugs were administered at a volume of 10 ml/kg, 15 minutes prior to the start of experimental protocols. These doses and drug delivery time course were selected based on previous literature[3,4,15].

2.3 Assessment of Mechanical Sensitivity

The von Frey test was performed as described previously[13,98] to assess mechanical sensitivity. Subjects were placed in a plexiglass chamber on top of a metal mesh floor for one hour a day over a period of three days to habituate to the enclosure prior to testing. On test day, mice were placed in the chambers and habituated for 30 minutes, or until cessation of exploratory behavior. An electronic von Frey device (Ugo Basile, Italy; Cat. 38450) was used to assess mechanical sensitivity, as it was capable of detecting lower thresholds typical of female mice[99]. The filament was applied to both left and right hind paws, for a total of six trials (3 per paw) until a sudden paw withdrawal, flinching, or paw lick was noted. Each trial was separated by 5 minutes and trials were averaged to assess mechanical sensitivity.

2.4 Assessment of Thermal Sensitivity

The Hargreave's test was performed as described previously[13,100] to assess thermal sensitivity. Briefly, subjects were placed in the same plexiglass chambers used for the von Frey test, placed on top of a glass platform. The mice habituated to the apparatus for one hour on the day prior to testing. On test day, mice were placed in the chambers and habituated for 30 minutes, or until cessation of exploratory behavior. A radiant heat source (IITC, Cat. 390) was applied to the plantar surface of the hind paw and the time to a nocifensive response was recorded, with a maximum exposure limit of 20 seconds to avoid tissue damage. Three trials were performed on both the left and right hindpaws, with at least ten minutes between each trial. All trials were averaged to obtain a measure of thermal sensitivity.

2.5 Assessment of Morphine Antinociception

Subjects were habituated for at least 2 days prior to each experiment. Mice received the GPR171 agonist (MS15203; 10mg/kg, s.c.), morphine (5mg/kg, s.c.), saline (10ml/kg, s.c.) and a combination of morphine and the GPR171 agonist – each delivered at least 72 hours apart in a counterbalanced design to ensure systematic variation. Prior to each drug treatment, mice were tested on the hot plate and tail flick tests to assess baseline nociception, and then were tested again 15, 30, 60, and 120 minutes following the drug treatment.

Nociception was assessed using the hot plate test and tail flick warm-water test as previously described[4,101]. In the hot plate test, mice were placed on a hot plate (Harvard Apparatus, Holliston, MA) heated to 50 degrees Celsius and the latency for mice to lick

their hindpaw was measured as a nociceptive response. In the tail flick warm-water test, mice were gently restrained and their tails were placed in a warm water bath (Thermo-Fisher, Waltham, MA) maintained at 52 degrees Celsius. The time taken for a mouse to flick their tail out of the water was measured. To avoid tissue damage, mice were removed if no response occurred within 60 seconds during the hot plate test or 20 seconds during the tail flick test.

Time course of antinociceptive response to individual drugs was calculated by plotting the mean of time to nociceptive response as a function of time. Antinociception was quantified in both the hot plate and tail flick as percentage of maximal possible effect (%MPE) in order to minimize any potential baseline differences between sexes. The %MPE was calculated as $[(T_1 - T_0)/(T_2 - T_0)]\times100$ where T_0 and T_1 were the time to nociceptive response before and after drug administration, and T_2 was the cut off time. Area under the curve (AUC) analysis was also calculated to assess overall response to each drug. AUC was calculated using the Trapezoidal method (as performed by Keyhanfar et al.[99]).

2.6 RT-qPCR

RT-qPCR was performed as described previously[13]. Naïve male and female mice were euthanized by cervical dislocation and decapitation (6 per genotype, per sex). Brains were removed, and the periaqueductal gray (PAG) was dissected and snap-frozen on dry ice. RNA was extracted from tissues using Trizol (Cat. 15596026, Invitrogen) and RNeasy Plus Mini Kit (Cat. 74136, Qiagen). RNA was quantified, and cDNA was synthesized using the Maxima first-strand synthesis kit for RT-qPCR (Cat. K1642,

Thermo-Fisher). Samples were prepared using SYBR green Universal Supermix (Cat. 1725121, Bio-Rad) and primers specific for GAPDH (housekeeping), ProSAAS, and genes that produce both opioid receptors and peptides that regulate pain responses were selected. Specifically, mu-, delta-, and kappa-opioid receptor genes as well of nociception receptor 1 were analyzed. Additionally, the genes involved in the production of endogenous opioids (PENK and PDYN) and Substance P (TAC1) were selected. Primer sequences are listed in Table 1. All primers were pre-designed by IDT DNA (IDT, Coralville, Iowa, USA) Gene expression analysis was done on a real-time thermocycler. The synthesized cDNA was assayed in triplicate. Results were analyzed using the $2^{-\Delta\Delta Ct}$ method in which $\Delta\Delta Ct = \{(Ct_{target\,:\,KO/HETsample} - Ct_{GAPDH\,:\,KO/HETsample}) - (Ct_{target\,:\,WTsample} - Ct_{GAPDH\,:\,WTsample})\}$ for each sex. Negative control reactions were performed to ascertain contaminant-free cDNA synthesis, and primer specificity was evaluated using melt curve analyses.

Table 1. Primer Sequences for RT-qPCR

Primer Target	Primer 1 Sequence (5'-3')	Primer 2 Sequence (5'-3')
Mu-opioid	CGGCTAATACAGTGGATCGAAC	CAACATGAGTCGGAGAAGGAT
Delta-opioid	GGTCTTGGCTTCAGGTGT	GCAGATCTTGGTCACAGTGT
Kappa-opioid	CATCACCGCTGTCTACTCTG	GTTGCGGTCTTCATCTTCGT
Nociceptin	TGCCTCGTCATGTATGTCATC	GGTAGCAGTCTTCATCTTGGTG
PENK	CTACAGTGCAGGCGGAAT	CTGTCCTTCACATTCCAGTGT
PDYN	TCTAATGTTATGGCGGACTGC	CATGTCTCCCACTCCTCTGA
TAC1	AGGCTCTTTATGGACATGGC	TCTTTCGTAGTTCTGCATCGC

2.7 Statistical analyses

Statistical analyses of behavioral data were generated by using one-way or two-way ANOVA, when appropriate, using Prism Software (version 10.0, GraphPad Software). Statistical analysis of morphine antinociception was evaluated using a mixed factorial ANOVA, using R. Post hoc tests were conducted to make pairwise comparisons, when appropriate. Statistical analyses of all RT-qPCR data was generated using two-way ANOVAs. The ROUT method[36] was used to identify and exclude outliers. Results are presented as mean ± standard error of the mean (SEM).

3. Results

3.1 GPR171 Knockout Does Not Alter Mechanical or Thermal Sensitivity

In order to assess the effect of GPR171 knockout on baseline mechanical and thermal sensitivity, naïve male and female WT, HET, and KO mice were tested on the von Frey and Hargreave's test. In the von Frey test (Figure 1B) males had a significantly higher nociceptive threshold compared to females ($F(1, 51) = 16.30$, $p < 0.0001$), but there were no differences between HETs, KOs, and WTs ($F(2, 51) = 1.98$, $p = 0.14$), or interaction between sex and genotype ($F(2, 51) = 0.63$, $p = 0.53$).

Similarly, in the Hargreave's test (Figure 1B) males had significantly higher nociceptive thresholds compared to females ($F(1, 51) = 25.37$, $p < 0.0001$), however, there was no significant effect of genotype ($F(2, 51) = 0.01$, $p = 0.98$) or interaction between genotype and sex ($F(2, 51) = 1.08$, $p = 0.34$), indicating that GPR171 is not necessary for normal thermal or mechanical sensitivity

Figure 1

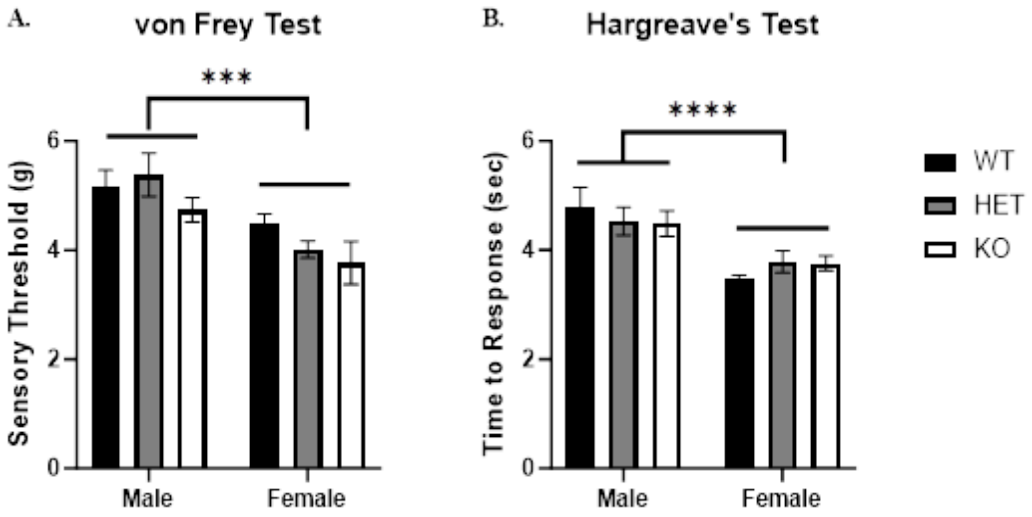

Figure 1. GPR171 KO Does Not Alter Mechanical or Thermal Sensitivity (A) WT males (n=7) and females (n=11) did not differ in mechanical sensitivity in the von Frey test compared to their HET (males, n=12; females n= 7) and KO littermates (males, n=10; females n= 10). **(B)** Similarly, thermal sensitivity was not altered by genotype in the Hargreave's test in the same cohort of mice as in (A). Analyses consisted of Two-way ANOVAs with Tukey's HSD post hoc tests, ***p<0.001, ****p<0.0001

Additionally, in the hot plate test, there were no baseline differences in thermal sensitivity between genotypes ($F(2, 49) = 0.52$, $p=0.59$; Figure 2A). There were also no differences in tail flick baseline responses between genotypes ($F(1, 49) = 1.36$, $p=0.24$; Figure 2B), further corroborating the results in the Hargreave's test that GPR171 is not necessary for normal thermal sensitivity.

Figure 2

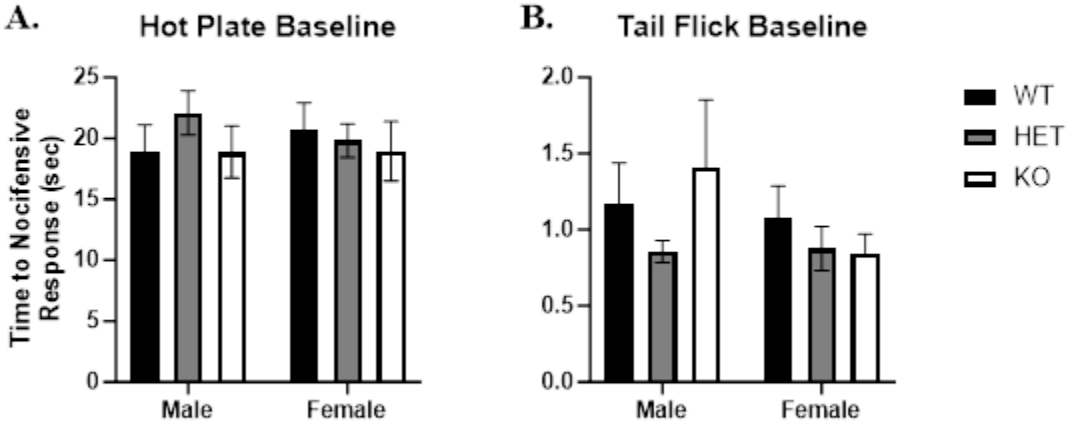

Figure 2. GPR171 KO Does Not Alter Baseline Thermal Sensitivity in the Hot Plate and Tail Flick Tests (A) WT males (n=9) and females (n=10) did not differ in thermal sensitivity in the hot plate test compared to their HET (males, n=10; females n= 12) and KO littermates (males, n=7; females n= 7). **(B)** Similarly, thermal sensitivity was not altered by genotype in the tail flick test in the same cohort of mice as in (A). Analyses consisted of two-way ANOVAs

3.2 GPR171 Knockout Reduces Morphine Antinociception

In the hot plate test, a mixed factorial ANOVA of %MPE data revealed a main effect of drug ($F(3, 882) = 30.58$, $p < 0.001$), and a significant effect of time ($F(4, 112.79) = 28.12$, $p < 0.001$), as well as drug over time ($F(12, 882) = 9.95$, $p < 0.001$), indicating that collapsed across sex and genotype, both morphine and the combination of morphine and the GPR171 agonist produced significant antinociception beginning at 15 minutes after injection (Tukey's HSD, $p < 0.05$). Interestingly, there was a significant interaction between sex and drug ($F(3, 882) = 7.46$, $p < 0.001$), with post hoc analyses revealing that morphine produced greater antinociceptive effects in females compared to males (Tukey's HSD, $p < 0.0001$). No other sex specific differences were detected within

other drug treatments (Tukey's HSD, $p > 0.05$). There was also a significant interaction between drug and genotype ($F(6, 882) = 2.64$, $p < 0.01$). Post hoc tests indicated that while the combination treatment was effective in reducing antinociception for all genotypes of both sexes (Tukey's HSD, $p < 0.05$), morphine treatment was not significantly different from saline for male KO mice (Tukey's HSD, $p > 0.05$) or female KO mice (Tukey's HSD, $p > 0.05$).

To better visualize comparison between groups, area under the curve (AUC) was calculated which takes into account both maximum effects and duration of action for each drug treatment[99]. Results from analysis of %MPE for hot plate data were corroborated by AUC analysis in which two-separate ANOVAs were conducted to evaluate the effect of drugs, by genotype, in males and females. In males, morphine produced antinociception in the WT, but not the GPR171 HETs or KOs compared to the saline treated controls (Tukey's HSD, $p > 0.05$; Figure 3A). However, the combination treatment was not significant in any genotype (Tukey's HSD, $p > 0.05$; Figure 3A). Females showed an increase in antinociception after morphine and combination treatment in both HET and WT mice (Tukey's HSD, $p < 0.05$; Figure 3B); however, in KO females, only the combination treatment, and not morphine alone, was effective in producing antinociceptive relative to the KO saline treated females (Tukey's HSD, $p < 0.05$; Figure 3B). Taken together, on the hotplate test GPR171 knockout reduced morphine antinociception in both male and female mice.

In the tail flick test, a mixed factorial ANOVA of %MPE data revealed similar main effects of drug ($F(3, 931) = 127.29$, $p < 0.001$), time ($F(4, 931) = 67.8$, $p < 0.001$), and drug over time ($F(12, 931) = 22.02$, $p < 0.001$), indicating that collapsed across sex

and genotype, both morphine and the combination of morphine and the GPR171 agonist produced significant antinociception beginning at 15 minutes after injection (Tukey's HSD, $p < 0.05$), as seen in the hot plate test. There was also an interaction between drug, sex, and genotype ($F(6, 931) = 1.96$, $p = 0.06$). Post hoc tests indicated that while the combination treatment was effective in reducing antinociception for all genotypes of both sexes (Tukey's HSD, $p < 0.05$), morphine treatment was not significantly different from saline for male KO mice (Tukey's HSD, $p > 0.05$) or female KO mice (Tukey's HSD, $p > 0.05$).

AUC analyses, by sex, revealed a similar pattern in tail flick test as the data in the hot plate test. However, in males, morphine produced antinociception in the WT, HETs, and KOs compared to the saline treated controls (Tukey's HSD, $p < 0.05$; Figure 3C). The combination treatment produced antinociception in male HETs and KOs (Tukey's HSD, $p < 0.05$; Figure 3C). Females showed an increase in antinociception after morphine and combination treatment in both HET and WT mice (Tukey's HSD, $p < 0.05$; Figure 3D); however, in KO females, neither treatment was effective in producing antinociceptive relative to the KO saline treated females (Tukey's HSD, $p < 0.05$; Figure 3D). These results indicate that GPR171 knockout did not affect morphine efficacy in male knockouts, but did for females in the tail flick test.

Figure 3

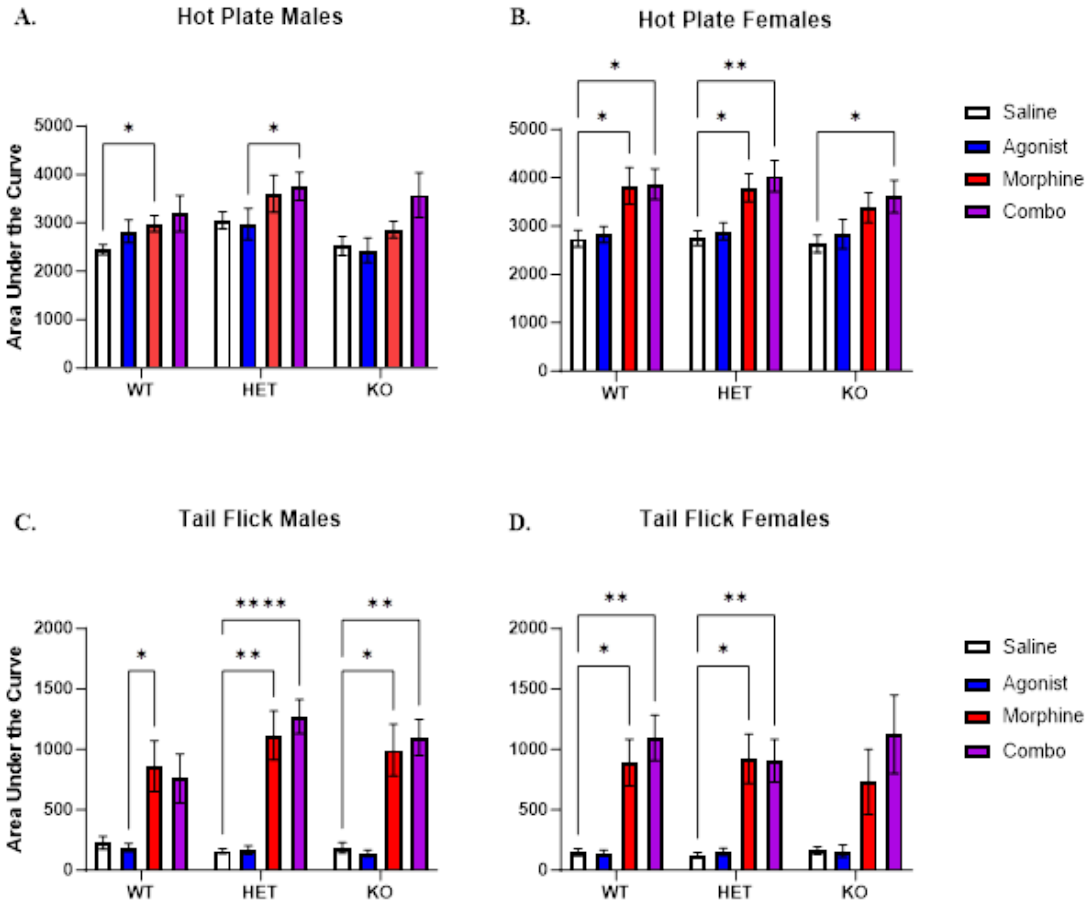

Figure 3. GPR171 Knockout Reduces Morphine Antinociception in a test and sex-specific manner. (A) Morphine increased antinociception in male WT mice (n=9) in the hot plate test, relative to WT saline-treated controls, but did not significantly increase antinociception in HET (n=10) and KO (n=7) males**. (B)** Morphine increased antinociception in female WT (n=10) and HET (n=12) mice, but not KO mice (n=7). The combination drug treatment was effective in all genotypes. **(C)** In the tail flick test, morphine increased antinociception in all male mice relative to agonist treatment (WT) or saline (HET and KO). **(D)** In the tail flick test, female WT and HET mice experienced increased antinociception with morphine and the combination treatment. Female KO mice did not experience significant antinociception with either treatment. No differences were noted between agonist-treated mice and saline-treated mice in any genotype, test, or sex. Analyses consisted of Two-way ANOVAs of AUC with Tukey's HSD post hoc tests, *p<0.05, **p<0.01, ****p<0.0001

3.3 GRP171 Knockout Alters Receptors and Peptides Involved in Pain Processing in the PAG

RT-qPCR was used to assess whether receptors or peptides involved in pain processing were altered between KO, HETs, and WT mice in the PAG. In females, a two-way ANOVA revealed a significant main effect of gene ($F(2, 39) = 12.21$, $p < 0.0001$) and interaction between gene and genotype ($F(4, 39) = 2.80$, $p < 0.05$). Additionally, in males there was a significant interaction between peptide and genotype ($F(4, 43) = 2.60$, $p<0.05$). Post hoc analyses reveal that specifically TAC1 expression pattern changed with genotype, with male KOs displaying upregulated gene expression relative to male HETs (Tukey's HSD, $p < 0.05$, Figure 4A), and female HETs displaying unregulated expression relative to female WT controls (Figure 4C, Tukey's HSD, $p < 0.05$).

There was no effect of genotype on the expression patterns of the receptors for female mice ($F(2, 57) = 0.98$, $p = 0.37$, Figure 4D), indicating that the knockout of GPR171 did not affect receptor gene expression in the PAG. However, in males there was a significant interaction in receptor and genotype in males ($F(6, 60) = 2.440$, $p < 0.05$) with post hoc tests revealing a significant downregulation of Delta opioid receptor gene in GPR171 KO mice relative to WT mice (Tukey's HSD, $p < 0.05$), and a significant upregulation of Mu opioid receptor gene in GPR171 KO mice relative to HET mice (Tukey's HSD, $p < 0.01$) as seen in Figure 4B.

Figure 4

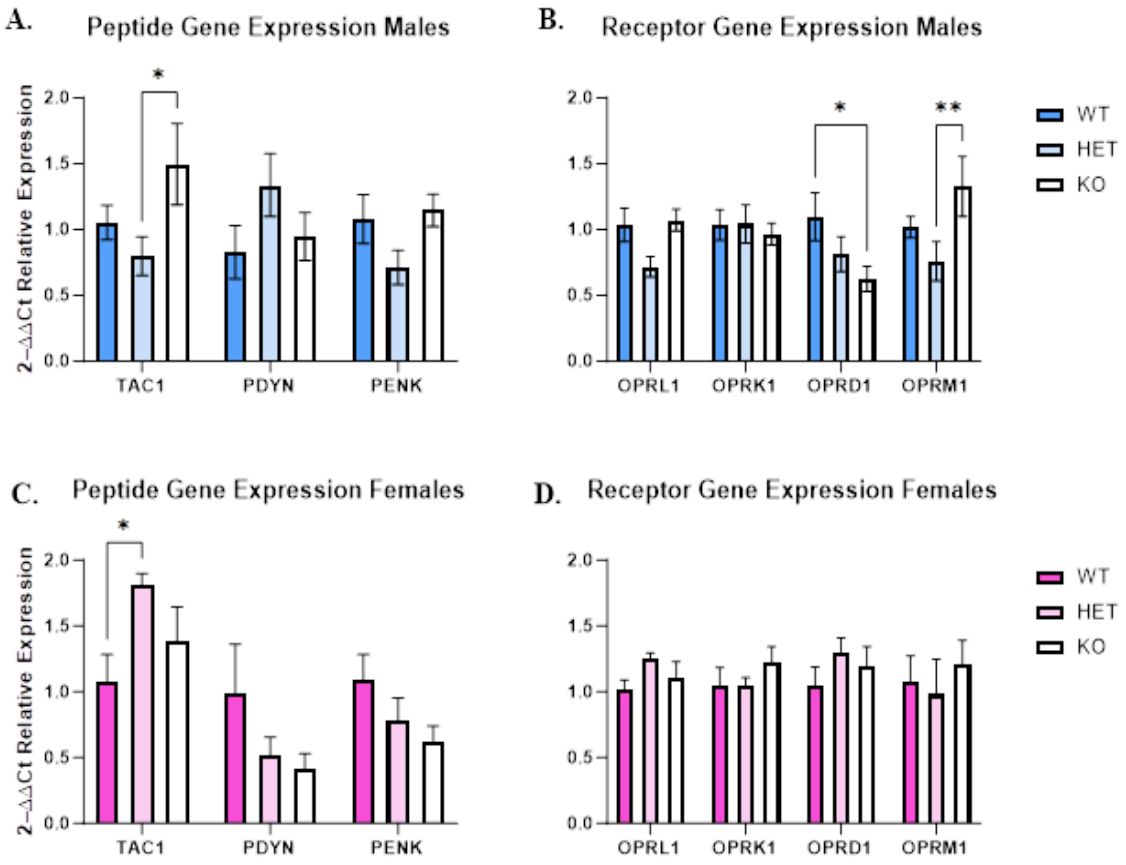

Figure 4. GPR171 Knockout Mice Have Altered Expression Levels of Genes Involved in Pain in the PAG. (A) Male knockout mice have increased expression of TAC1 relative to HET males, but not compared to WT males. No other peptide producing genes were significantly altered. (B) Genes that produce receptors involved in the pain response were altered in males. The delta-opioid receptor gene was downregulated in knockout, while mu-opioid receptor gene was upregulated. (C) TAC1 was upregulated in female HETs. No other peptide producing genes were altered. (D) No genes for receptors displayed altered expression in females between genotypes. All groups consisted of n=6. Analyses consisted of Two-way ANOVAs with Tukey's HSD post hoc tests, *$p<0.05$, **$p<0.01$

4. **Discussion**

In this study, we demonstrate that GPR171 is necessary for normal morphine antinociception but is not required for normal thermal and mechanical sensitivity. We show that while females are more sensitive to both mechanical and thermal stimuli – a result well documented in the literature[102,103], their sensitivity is not dependent on genotype. Despite GPR171 not being necessary for normal baseline sensitivity, GPR171 does seem to be necessary, at least in part, for normal morphine antinociception. Notably, GPR171 knockout mice did not display significantly increased morphine antinociception, though these results were dependent on test and sex. Additionally, our study reveals that GPR171 knockout induces alterations in several genes that regulate the pain response in a sex specific manner, with male knockouts displaying significantly more alterations than female knockout mice.

Previous research has shown that systemic injection of a GPR171 agonist or antagonist did not alter tail flick or hot plate latencies, revealing that GPR171 does not play a vital role in changing baseline nociception when manipulated pharamacologically[4]. In addition chronic treatment of the GPR171 agonist did not alter baseline thermal or mechanical sensitivity[13]. Our results further validate these findings by showing that the genetic deletion of GPR171 does not alter thermal or mechanical sensitivity or baseline nociception on the hot plate and tail flick tests. Interestingly, while GPR171 is expressed in small diameter neurons, mostly comprised of C- and/or Aδ-fiber nociceptors[8] which transmit pain and mechanical touch signals from the periphery[104], the results of this study suggest that GPR171's role in these nociceptors is unlikely to be a necessary component of baseline pain and touch signaling.

While GPR171 may not be critical for the initial sensory perception of pain and touch sensitivity, its role becomes pronounced in the modulation and amplification of pain signals, particularly in the presence of morphine. In our study, we show that on the hot plate test GPR171 knockout resulted in reduced morphine antinociception in both males and females. In the tail flick test, while genotype had minimal impact on morphine antinociception in males, female GPR171 KO mice experienced reduced morphine antinociception relative to the saline treated controls. Test differences between the tail flick and hot plates tests, such as observed here, have been noted in previous studies, with tail flick results generally more exaggerated than hot plate results[4,15]. This is likely due to the mechanisms involved in each test, as the hot plate is considered a measure of supraspinal responses to pain which involves the coordination of several different brain areas, while the tail flick measures spinal reflexive pain[105]. Regardless of tests differences, the overall significant interaction between drug and gene that we show, particularly the diminished efficacy of morphine in GPR171 knockout mice, highlights GPR171's potential as a modulatory node in pain analgesia, possibly influencing opioid receptor dynamics in supraspinal areas and downstream signaling mechanisms in the spinal cord. This is the first study to show that GPR171 is necessary for normal morphine antinociception *in vivo*. Previous research has shown that the knockdown of GPR171 in cell culture reduced signaling by DAMGO, but not Deltorphin II, indicating the mu-opioid signaling – through which morphine works best – was decreased in the absence of GPR171 *in vitro*[4]. Additionally, pharmacological blockade of GPR171 via systemic antagonist injection resulted in decreased morphine efficacy[4], further validating the importance of GPR171 signaling in the efficacy of opioids.

One potential mechanism to explain the connection between GPR171 activity and morphine efficacy is that mu-opioid receptors and GPR171 are thought to form a heterodimer in which they functionally interact with each other[4,15]. While there is no direct evidence for this, both mu opioid receptors and GPR171 have been shown to interact with other receptors in a heterodimeric fashion[14,96]. Additionally, similar to our results, when one functional part of a mu receptor heterodimer was knocked out, changes in morphine antinociception ensued[106–108]. Interestingly, previous research has supported the mu-GPR171 heterodimerization hypothesis with evidence that GPR171 agonist paired with morphine produced greater antinociception than either alone[4,15]. In our study we found the combination drug treatment was still partially effective at producing antinociception, even in GPR171 knockout mice. However, the results were inconsistent across test and sex. Regardless, these results argue that the combination drug may be signaling through alternate off-site targets, though further research is needed to validate this.

Sex differences were apparent in both behavior and gene expression in our study. In our study, antinociceptive effects of morphine were greater in females on the hot plate test, but not on the tail flick test, relative to males. Generally, males, with increased mu-opioid receptor availability and binding in the PAG, have greater antinociceptive responses to morphine compared to females[109]. However, this effect is dependent on a variety of conditions, and is not always replicated[110]. One potential explanation of the observed sex difference in morphine antinociception is the influence of stress-induced analgesia, a common effect found with repeated testing, as done in our study[111]. Stress-

induced analgesia can affect females more than males[112], leading to greater baseline analgesia, and can potentially explain our results. Interestingly, in the hot plate test, repeated testing had minimal effect of baseline latency and did not impact morphine antinociception[111], but these results have not been shown in tail flick test, which could explain why results were found in one test and not the other.

We observed other sex differences between males and females in our study that were genotype specific. In particular genes that produce opioid receptors, specifically the delta- and mu- opioid receptors were altered in male knockout mice, but not in females. Additionally, TAC1, the gene that produces Substance P, was upregulated in HET females and male KOs. The upregulation of mu-opioid receptors in males likely explains why male knockouts experienced antinociceptive effects with morphine in the tail flick and female knockouts did not. Previous research has identified that PAG mu opioid receptor activation is a driving force in sex differences in antinociception in the tail flick test[113]. In addition to changes in mu-opioid receptor expression, the delta-opioid receptor was downregulated in male KO mice in our study. The specific downregulation noted here is particularly interesting because signaling via the delta receptor was not affected by GPR171 knockdown in cells, unlike mu-opioid signaling[4]. While delta-opioid receptors are located in the PAG and have modulatory roles in pain and analgesia[114], their specific role is relatively unexplored and represent an open avenue for future research. Finally, the alterations to TAC1, may represent a compensatory genetic alteration to maintain homeostatic balance of pain processing that is diminished in GPR171 knockouts. Previous research has shown that injecting TAC1 in the PAG increases antinociception in the hot plate test[115–117]. However, this increase in TAC1 was not

significant enough to compensate for the loss of morphine antinociception in GPR171 knockouts. These insights are confined to the PAG, a crucial but not exclusive region in pain processing, suggesting that a broader examination across multiple pain-related regions might reveal additional aspects of morphine's action and GPR171's role in antinociception. In specific, further research on spinal-mediated mechanisms of pain processing and supraspinal mediated mechanisms will further clarify the roles of GPR171 and test-dependent differences we noted in our study.

This study explored the role of GPR171 in acute pain - it does not address the roles of GPR171 in any other pain state including chronic or inflammatory pain. Previous work has shown that the GPR171 agonist, alone, is capable of alleviating these pain states in a sex and time-dependent manner[8,13]. As such future studies should evaluate whether GPR171 knockout leads to alterations in more chronic pain states. Additionally, hormonal factors, including the influence of estrogen, warrant further investigation, as GPR171 activity is influenced by estrogen (as shown in Chapter 2). Due to the extensive number of groups, analyzing estrogen dependent differences within our studies would have resulted in samples sizes too small to produce meaningful results.

In conclusion, our study highlights the critical role of GPR171 in enhancing morphine antinociception. The decreased morphine antinociceptive caused by the genetic deletion of GPR171 underscores the essential role of GPR171 in augmenting opioid-induced antinociception, possibly offering a pathway to enhance pain relief. The observed genotype and sex differences emphasize the need for a nuanced approach to pain management, considering the genetic background and sex of the individual. While GPR171 is only beginning to be studied in human conditions[88,118], this study highlights

the need for future research to delve deeper into the mechanisms underlying GPR171's action and explore its therapeutic potential in combination with opioids to offer a more effective and less addictive pain management strategy.

5. Summary

The creation of new genetic models via CRISPR gene editing has allowed for the discovery of several “pain genes” that regulate pain or analgesia that can be exploited as pharmacological targets and reveal critical components of pain signaling[119]. Here, we explored whether GPR171 could be considered a pain gene and found that while genetic deletion of GPR171 did not alter baseline thermal or mechanical sensitivity, it was necessary for normal morphine antinociception. This was in part due to genetic compensatory changes in the PAG with other pain-related genes. Together our study validates ongoing research into pharmacological agents targeting GPR171 for pain relief in combination with other analgesic compounds.

CHAPTER V

GENERAL DISCUSSION

The aim of this dissertation was to elucidate the roles of GPR171, both known and novel, with a specific focus on sex differences in receptor function. I utilized a comprehensive approach that integrated pharmacological methods, genetic knockouts and opioid interactions to expand on the current understanding of GPR171's impact on physiological and behavioral processes. Key findings show that GPR171 antagonism reduces anxiety in female mice, with significant interactions observed between GPR171 and estrogen, highlighting the receptor's role in modulating sex-specific responses. Additionally, I used a newly developed GPR171 knockout mouse that allowed me to explore the necessity of GPR171 in both new and existing functions of the receptor and discovered that GPR171 was necessary for normal physiological and mood-related behaviors, though results were highly dependent on sex. Finally, I show that GPR171 is necessary for normal morphine antinociception. Importantly, my results consistently underscored significant sex-dependent effects, suggesting that GPR171 is a potent modulator of sex differences across various behavioral paradigms.

1. Main Findings

This dissertation has uncovered pivotal roles for GPR171 in the regulation of anxiety and mood behaviors, with significant variations noted between sexes. In Chapter 2, the use of the GPR171 antagonist resulted in a reduction in anxiety-related behaviors in female mice, a finding that adds to the previous research solely conducted in males[3]. I

showed that this anxiolytic function of the GPR171 antagonist was abolished in mice that underwent a subchronic stress paradigm, indicating a functional relationship between stress and GPR171 activity. Critically, I showed that the GPR171 agonist actions were dependent on estrogen levels and that this interaction with estrogen implies a hormonal influence on GPR171's activity, supporting its potential as a target for mood disorders that exhibit a strong sex bias.

In Chapter 3, I used the newly developed GPR171 knockout mouse model to reveal that GPR171 is crucial for maintaining normal physiological functions such as feeding and drinking behaviors. Male knockout mice showed increased food intake and decreased water consumption, confirming GPR171's hypothesized role in hunger and thirst regulation. These findings suggest that GPR171 could be a key regulator within the neural circuits controlling homeostatic and motivational aspects of feeding. Additionally, GPR171 male knockout mice displayed increased muscle incoordination – a behavior that has not been explored in previous literature on the receptor functions. Chapter 3 also investigated the effect of GPR171 knockout on mood and found that the knockout reduced anxiety-like behaviors in males, as was seen in previous pharmacological studies[3], but not in females. Interestingly, the majority of findings were not evident in female mice, accentuating the sexually dimorphic effects of GPR171 found previously[13] and in Chapter 2.

One of the most compelling discoveries of this dissertation is the essential role of GPR171 in enhancing the efficacy of morphine antinociception, especially notable since this effect was consistently sex-dependent. The reduction of morphine efficacy in knockout mice (as shown in Chapter 4) provides a novel insight into the opioid pain

management system, indicating that GPR171 is necessary for normal morphine antinociception, but it is not necessary for normal thermal or mechanical sensitivity. This suggests that GPR171 could potentially be a biomarker for identifying or predicting sensitivity to opioid treatment, and validates previous research approaches that have aimed to enhance morphine efficacy by pairing it with the GPR171 agonist[4].

Finally, an overarching critical discovery that I emphasize throughout our studies is the interaction between GPR171 and sex, which highlights the necessity of considering sex as a biological variable in pharmacological research and treatment. The sex-dependent effects observed in GPR171 knockout mice, particularly in morphine antinociception, mood, and consumption support the hypothesis that GPR171 may act differently in male and female physiology due to hormonal influences as we demonstrate in Chapter 2, or may be region and test specific, as we discuss in Chapter 3.

2. Theoretical and Practical Implications

This dissertation not only contributes to the field of GPR171 research but also broadens the current understanding of G-protein-coupled receptors (GPCRs) in general. Traditionally, GPCRs are studied in narrow contexts, often linked to one or two specific behaviors, leading to rapid drug development focused on these limited functions. However, my work suggests that GPCRs, including GPR171, should be considered across a wider range of behaviors that correspond to their known brain expression patterns before being targeted for specific disorders. This approach could help anticipate potential off-target effects and guide the development of more precise therapeutics.

More specifically, my work supports the general field of ProSAAS research. ProSAAS is one of the most abundant peptides in the brain and is produced by the gene PCSK1N located on the X chromosome. It generates various peptide products, including BigLEN and PEN, which often exhibit opposing functions, suggesting that these peptides finely tune the balance of neural activity. While no study has directly explored the differential expression of ProSAAS between males and females, there are likely variations in the endogenous tone of ProSAAS and its derivatives that could be a fundamental factor driving the sex differences observed in studies of GPR171.

GPR171, in particular, may be a valuable genetic marker for various diseases and disorders. Alterations in GPR171 expression could indicate increased vulnerability to conditions, including those beyond the traditionally studied domains. Although research into GPR171 in human populations is scant, its association with cancer suggests that it may be implicated in a range of other human diseases not evaluated here. As studies transition from preclinical to clinical settings, GPR171 could emerge as a crucial target for developing treatments that are fine-tuned to the complexities of human pathology.

3. Limitations and Future Directions

I acknowledge several limitations in my work that highlight areas for future exploration. One significant constraint is the potential for genetic compensatory mechanisms that can occur with any knockout mouse model[120] to potentially dampen the effects of GPR171 knockout. However, I found that even in situations in which compensatory changes to receptors did occur (as seen in Chapter 4), the changes were not enough to obscure the effects of GPR171 deletion. My research also corroborated

research with genetic knockdowns[2] and pharmacology[4] which are not as subject to compensatory changes[121]. Additionally, while I explored a variety of behaviors in which GPR171 was involved, the creation of the GPR171 knockout opens up avenues for the exploration of an even more comprehensive list of behaviors in which GPR171 may be necessary. Finally, I provide extensive evidence for sex-specific alterations in behaviors mediated by GPR171, however I cannot conclusively provide a mechanism for these differences, as this study's primary focus was to establish phenotypic differences for future research to expand upon.

As such, there is a great deal of future directions for discovering and expanding on GPR171 functions. Other behaviors are likely to be affected by GPR171 knockout including chronic pain states, which have been shown to be mediated with GPR171 drugs[13]. Additionally, as GPR171 is involved in the regulation of T cells[85], future research should consider the impact of GPR171 deletion in immune and inflammatory signaling, particularly within the contexts in which the receptor has already been shown to function. Of note, while this dissertation offers a neurobehavioral overview of the functions of GPR171, receptor dynamics and signaling pathways need to be clarified and uncovered that could directly impact or provide a mechanism to explain these functions. While GPR171 has been shown to signal through Gi/o-mediated pathways it is possible that this is not consistent with females as other sexually dimorphic receptors such as CRF1 are biased towards different pathways in males compared to females, driving the sex differences caused by receptor activation[122]. In addition, specific GPR171-mediated circuit mechanisms also warrant future consideration. While GPR171 is proposed to modulate activity in circuits from the NAc to the Hypothalamus, and activity in the VTA,

PAG, and BLA, other region-specific functions and circuits have yet to be explored.

4. Conclusion

In this dissertation, I sought to understand and validate the roles of GPR171 in health and disorder. I discovered the necessity of GPR171 in regulating anxiety behaviors and morphine antinociception using a newly created genetic GPR171 knockout mouse, and worked to directly compare males and females. These results advance our understanding of the overall roles and functions of GPR171, and a target for pharmacological manipulation in males and females for the treatment of multiple conditions. In specific, I set a foundation for further exploration into GPR171's mechanisms and its potential as a target for sex-specific therapeutic strategies in treating mood disorders and pain.

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PEER-REVIEWED PUBLICATIONS

McDermott M.V., Ram A., Mattoon M., Haderlie E., **Raddatz M.C.,** Thomason M., Bobeck E.N. (2023). A small molecule ligand for the novel pain target GPR171 shows minimal abuse liability in mice. *Pharmacology, Biochemistry, and Behavior, 224,* 173543

MANUSCRIPTS IN PREPARATION

Raddatz, M.C., Porter, C., Stott, M., Bobeck, E.N. (*in prep*). GPR171 is necessary for normal morphine antinociception without altering baseline mechanical or thermal sensitivity. *Preparing to submit to Genes, Brain and Behavior in May 2024.*

Raddatz, M.C., Porter, C., McDermott, M.V., Stott, M., Campbell, C., Bobeck, E.N. (*in prep*). GPR171 is necessary for normal physiological functions and mood-related behaviors in males, but not females. *Preparing to submit to Genes, Brain and Behavior in May 2024.*

Raddatz, M.C., Porter, C., Mattoon, M., Lauer, A., Bobeck, E.N. (*in prep*). The GPR171-BigLEN system regulates anxiety behaviors in female mice and is influenced by estrogen. *Preparing to submit to the Journal of Behavioural Brain Research in May 2024.*

Raddatz, M.C. & Freeman, S.M. (*in prep*). Teaching with desirable difficulty: Promoting student success in neurophysiology through the practice of productive struggle. *Final writing phase. Planning to submit to the Journal of Undergraduate Neuroscience*

Education in June 2024.

Bobeck, E.N., Fakira, A., Fricker, L.D., **Raddatz, .C.**, Morgan, D. (*in prep*). New insights towards the function of ProSAAS-derived peptides. *Finalizing writing. Planning to submit to the Journal of Pharmacology and Experimental Therapeutics in May 2024.*

Wagge, J., … **Raddatz, M.C.**, … & Lazarevic, L. B., (*under review*). Collaborative registered replication of Greskevicius et al.: Can pro-environmental behavior be promoted by inducing status competition?

AWARDS AND HONORS

2024	USU Legacy of Utah State Robins Award
2024	CEHS Legacy of Utah State Award
2024	Psychology Legacy of Utah State Award
2024	Psychology Graduate Student Teacher of the Year
2024	International Brain Awareness Week Grant
2023	USU Graduate Student Travel Award
2023	USU Psychology Student Researcher Travel Award
2023	Psi Chi Graduate Research Grant
2023	USU CEHS Graduate Student Research Award
2023	USUSA Graduate Enhancement Award
2023	ASPET Travel Award
2023	Graduate Research and Creative Opportunities Grant
2023	USU Community-Engaged Student Presidential Award (Runner Up)
2022	Biology Graduate Student Association Travel Award
2022	ADinstruments Educator Award
2018	USU CEHS Graduate Student Research Award

CONFEREFENCE PRESENTATIONS

Raddatz, M.C., Porter, C., Stott, M., & Bobeck, E. N. (2023, November). Mice lacking GPR171 displays alterations in mood in a sex-dependent manner. Poster presented at the Society for Neuroscience Conference, Washington D.C.

Raddatz M. C. & Freeman, S. M. (2023, August). Teaching with Desirable Difficulty: Promoting Student Success in Neurophysiology through the Practice of Productive Struggle. Oral presentation presented at Empowering Teaching Excellence Conference, Logan, UT.

Raddatz M. C. & Freeman, S. M. (2023, July). Teaching with Desirable Difficulty: Promoting Student Success in Neurophysiology through the Practice of Productive Struggle. Poster presented at the Faculty for Undergraduate Neuroscience Workshop, Bellingham, WA.

Raddatz M. C. & Bobeck E. N. (2023, May). Acute and Subchronic GPR171 Agonism Affects Anxiety and Depression in Female Mice. Poster presented at the American Society for Pharmacology and Experimental Therapeutics Conference, St. Louis, MO.

Raddatz M. C., Porter, C., & Bobeck E. N. (2023, January). The Role of GPR171 in Depression in Females: Exploring Hormonal Influences. Poster presented at the Winter Conference on Brain Research, Snowbird, UT.

Raddatz M. C., Mattoon M., & Bobeck E. N. (2022, November). The Role of GPR171 in Anxiety and Depression in Female Mice. Poster presented at the Society for Neuroscience Conference, San Diego, CA.

Raddatz M. C., Bobeck E. N., & Freeman S. M. (2022, November). Neuroscience Unwrapped: Creating a Sustainable Outreach Program at Utah State University. Poster presented at the Society for Neuroscience Conference, San Diego, CA.

Herron M., **Raddatz M. C.**, & Buhusi M. (2020, December). The Effects of Estrogen and Orexin on Latent Inhibition. Poster presented at Utah State University Fall Student Research Symposium, Logan, UT.

Raddatz M. C., Jensen K., Petersen M., Rasmussen V., Higgins H., & Martin S. C. (2018, May). A New Pathway to Awe: A Pilot Study. Poster Presented at the 40th Annual Association for Psychological Science Convention, San Francisco, CA.

Petersen M., **Raddatz M. C.**, Jensen K., Rasmussen V., Higgins H., & Martin S. C. (2018, May). Redefining Awe and Elevation: A Pilot Study. Poster Presented at the 40th Annual Association for Psychological Science Convention, San Francisco, CA.

Raddatz M. C., Armitage E., Taylor E., Ensign H. M., Mackintosh M. J., & Wiggins B. (2017, December). Replication of Tentori, K., Crupi, V., & Russo, S. (2013) On the Determinants of the Conjunction Fallacy. Poster presented at Brigham Young University-Idaho Fall Semester Research and Creative Works Conference, Rexburg, ID.

MENTORING EXPERIENCE

Fall 2023-Current	Colton Campbell, Mallory Wootton
Summer 2023	Cesia Ulloa; Summer Undergrad Research Grant Recipient
2022-Current	Mitchel Stott, Emmaline Harderlie
Spring 2023	Ryan Thalman
2022-2023	Christen Ellis; Undergraduate Research Grant Recipient
2021-2022	Sophia Mouritsen, Sydney Geisler, River McKinlay
2020-2021	Benjamin Steadman
Spring 2020	Todd Zimmerman
2019-2021	Michael Herron; Undergraduate Research Grant Recipient
2019-2020	Daryn Short
Summer 2019	Igor Neves

TEACHING EXPERIENCE

Instructor of Record: Utah State University

PSYC 3460 Neuroscience I	Summer 2020, Online Fall 2020, Online Spring 2021, Online Summer 2021, Online Spring 2023, Face to Face(F2F) Summer 2023, Online
BIOL 5625 Neurophysiology Lab (Co-Instructor)	Fall 2022, F2F Fall 2023, F2F

Lab Instructor: Utah State University

PSYCH 4420 Cognitive Psychology	Spring 2019, F2F

	Spring 2020, Hybrid
PSYCH 3450 Sensation and Perception	Fall 2019, F2F

Graduate Teacher's Assistant: Utah State University

PSYCH 3460 Neuroscience I	Spring 2020, Online
	Fall 2021, F2F
	Spring 2022, F2F
PSYCH 1400 Analysis of Behavior	Fall 2018, F2F
PSYCH 5330 Principles of Psychological Measurement	Fall 2018, Online
PSYCH 3500 Psychology Research Methods	Spring 2024, Online

Undergraduate Teacher's Assistant: Brigham Young University-Idaho

PSYCH 342 Abnormal Psychology	Fall 2017, F2F
PSYCH 302 Research Methods Writing Instructor	Spring 2017, F2F
	Summer 2017, F2F
	Fall 2017, F2F

PROFESSIONAL AFFILIATIONS

2023-2024	Member, ASPET Mentoring Network
2022-Current	Member, American Society for Pharmacology and Exp. Therapeutics
2022-Current	Member, Society for Neuroscience
2022-Current	Member, Psi Chi International Honor Society
2022-Current	Member, Faculty for Undergraduate Neuroscience
2022-Current	Member, Endocrine Society
2016-2017	Member, Association for Psychological Science

PROFESSIONAL SERVICE

2024	Science Fair Judge, Thomas Edison Charter School, Logan, UT
2023	Judge, Student Research Symposium, Utah State University
2023	Ad hoc Reviewer: *Neuroscience Letters*
2022	Judge, Biology Undergraduate Research Symposium, USU
2022	Editorial Board Member, *Curiosity: The Undergrad Journal of USU*
2022	Session Moderator, Utah Conference on Undergraduate Research
2021	Grant Reviewer, USU Undergraduate Research & Creative Grant
2019-present	Neuroscience Outreach Coordinator, Utah State University
2019	Neuroscience Outreach Volunteer, Utah State University