Prokineticin Receptor 2 Expressing Neurons of the Posterior Amygdala and Their Role in Reproductive Physiology

by

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Dedication

To my parents, Rafael and Guadalupe. Your sacrifices have opened many doors for me. Your life journey is a constant reminder that quitting is never an option. La vida me dio unos padres maravillosos. Gracias por tanto amor. ¡Si se pudo!

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Abstract

The reproductive neuroendocrine axis is tightly regulated by feedback loops controlling appropriate hormone levels for sexual maturation and reproductive success. Neuronal inputs from physiological processes that can affect reproduction (e.g., metabolism and sleep) converge to stimulate gonadotropin-releasing hormone (GnRH) neurons. Pulsatile GnRH release stimulates the pituitary biosynthesis and secretion of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Gonadotropins in turn stimulate the gonadal steroidogenesis and gametogenesis.

During development, GnRH neurons must migrate from the olfactory epithelium to the hypothalamus. Deficits in this developmental process results in Kallmann syndrome (KS). KS is characterized by GnRH deficiency and impaired or complete loss of sense of smell (hyposmia or anosmia, respectively). The prokineticin 2 and prokineticin receptor 2 (PROK2/PROKR2) system has been identified as a key component in migratory process of GnRH neurons. In fact, mice with global deletion of *Prokr2* replicate the phenotype of KS patients by displaying olfactory bulb dysgenesis and significant decrease in number of GnRH neurons in the hypothalamus. Though PROK2/PROKR2 system has been implicated in this developmental process, many aspects remain unclear and knowledge on neuronal networks of the PROK2/PROKR2 system is limited.

Aside from a developmental role, *Prokr2* is widely expressed in many reproductive control sites. Our lab generated a ProkR2-Cre mouse model allowing for functional studies and the neural circuitry. We hypothesized that ProkR2 expressing neurons have a role in adult reproductive function. To test our hypothesis, we set out to determine if ProkR2-Cre cells co-express gonadal steroid receptors relevant for reproductive success (estrogen receptor alpha and the androgen receptor). We found that the only brain site co-expressing Prokr2 and either receptor is the posterior amygdala. Building on this, we described the projection pattern of ProkR2-Cre neurons in the posterior amygdala of male and female mice using an adeno-associated viral vector (AAV) for molecular mapping. We found projections to several reproductive control sites such as the lateral septum, the medial preoptic area and the ventral premammillary nucleus. Lastly, we did a series of functional studies (i.e., sexual behavior, response to opposite and same sex odors, chemogenetic activation) to uncover the role of ProkR2 expressing neurons in the posterior amygdala. We found these neurons are highly responsive to opposite sex odor and have a role in opposite sex recognition in males. We also found that chemogenetic activation of these neurons can induce luteinizing hormone secretion. Our findings reveal that the Prokr2-expressing neurons of the posterior amygdala are part of a neuron circuitry that can sense the gonadal steroid milieu, integrate olfactory cues and modulate the neuroendocrine responses.

Chapter 1 Introduction

Recent estimates by the World Health Organization state that one in six people, globally, have experienced infertility at some stage in their life [1]. Infertility is defined as a couple's inability to get pregnant during a 12-month period of unprotected sex [2]. In the United States, it is estimated that about 9% of men and 11% of woman of reproductive age are affected by infertility [3]. Today, couples that are diagnosed with infertility have access to treatments and technologies that can result in successful pregnancy. In other cases, couples are diagnosed with idiopathic infertility, a clinical condition where the cause of infertility remains unknown. It is estimated that 15-30% of couples are diagnosed with idiopathic infertility [4-7].

Control of reproduction in mammals relies on a complex network of inputs from hypothalamic neurons that converge to regulate the activity of gonadotropin-releasing hormone (GnRH) neurons. GnRH neurons of the hypothalamus release GnRH peptide in a pulsatile manner to stimulate the pituitary for biosynthesis and secretion of gonadotropins, i.e., luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Gonadotropins then stimulate, via peripheral circulation, the gonads for steroidogenesis (androgens, estrogens, and progesterone) and gametogenesis (oocyte and spermatozoa). Through a feedback mechanism, gonadal steroids regulate release of reproductive hormones at the hypothalamic and pituitary level (Fig. 1.1) [8-14]. Proper development of all components of the HPG axis is critical for reproductive function. Abnormal development of the HPG axis results in reproductive hormone deficiencies or abnormal hormone profiles affecting fertility.

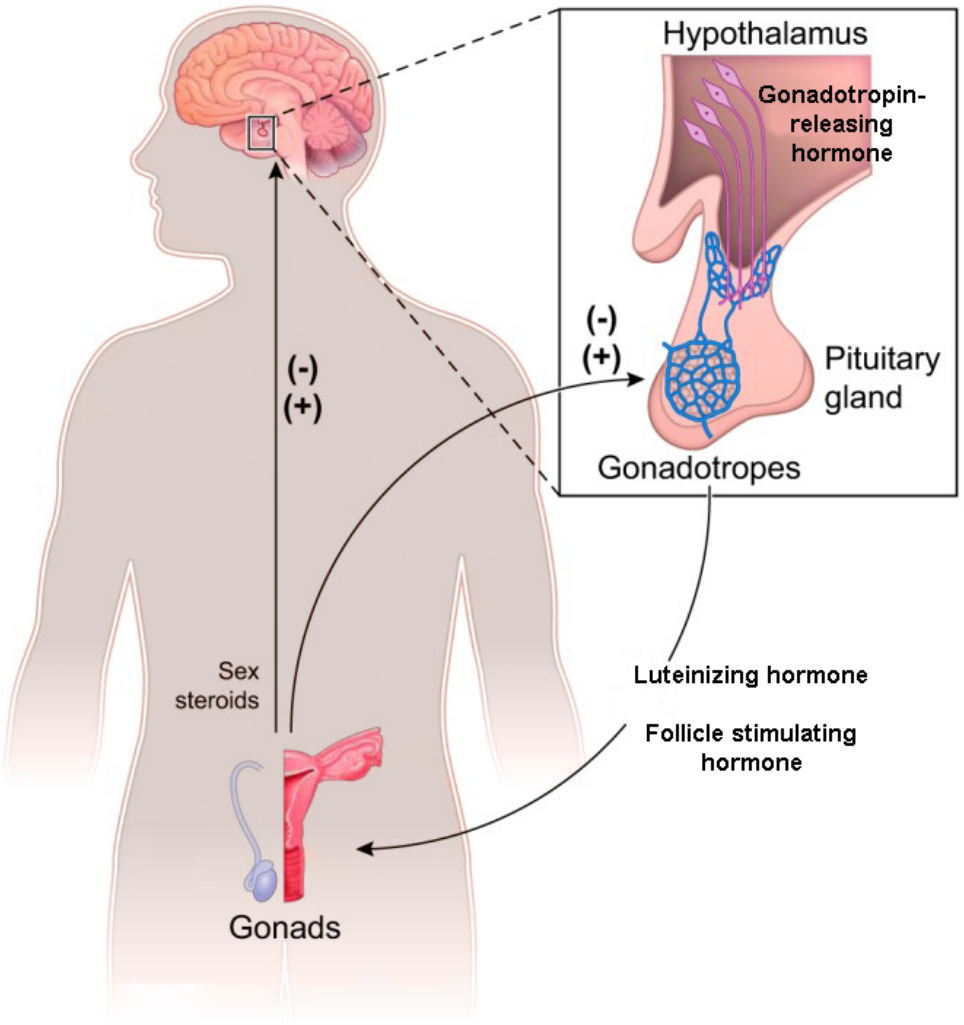

Figure 1.1: Schematic illustration of the hypothalamo-pituitary-gonadal (HPG) axis. GnRH neurons release GnRH that stimulates the anterior pituitary gland to synthesize and secrete gonadotropins (luteinizing hormone and follicle stimulation hormone). Gonadotropins stimulate the gonads for synthesis and release of sex steroids. Sex steroids regulate upstream reproductive hormones via feedback loops (positive feedback mechanism applies to females only). *Modified from Hill and Elias, 2018* [94].

Reproductive dysfunction can arise from abnormal migration and development of GnRH neurons. Approximately 1,500 GnRH neurons are found in the preoptic area and the caudal hypothalamus (in primates) and project to the median eminence where GnRH is released [15-17]. However, GnRH neurons have origins outside of the brain (Fig. 1.2). GnRH precursor cells arise in the olfactory placode and migrate, via olfactory axons, along the olfactory epithelium through

the cribriform plate. They progress through the olfactory bulb and move along the tract to reach the medial preoptic area of the hypothalamus [18-21]. GnRH cells leave the nasal placode around embryonic day 11 and their migration journey continues through the cribriform plate at embryonic day 16.5 [22-23]. Successful migration is necessary for normal reproductive function [24-25].

1.1 GnRH neurons and Congenital Hypogonadotropic Hypogonadism

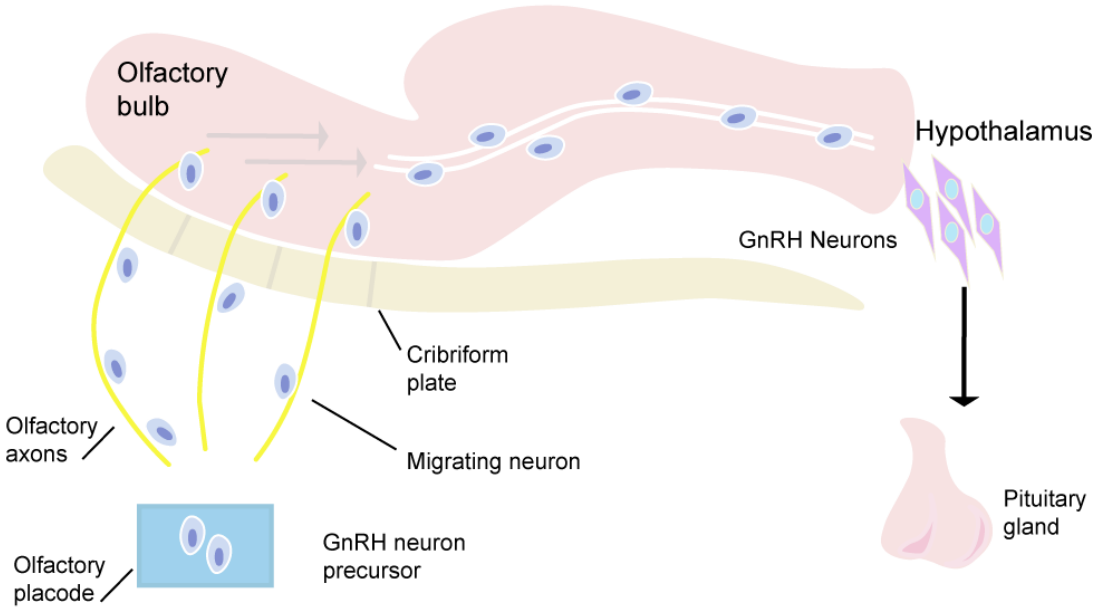

Figure 1.2: Schematic illustration of GnRH neuron precursor cells and their migratory pathway. GnRH neuron precursor cells originating in the olfactory placode migrate along olfactory axons through the cribriform plate and olfactory bulb to the medial preoptic area. *Modified from* Crowley, 2011.

Congenital hypogonadotropic hypogonadism (CHH) are diseases characterized by GnRH hormone deficiency resulting in incomplete sexual development and/or infertility [26-28]. It is

estimated that 1:8,000 men and 1:40,000 women are affected by this condition [28]. CHH is divided in three subtypes: syndromic forms of CHH (with additional non reproductive phenotypes e.g., cardiovascular defects, cleft/lip palate), isolated gonadotropin deficiency (IGD), and Kallmann syndrome (KS). CHH coupled with normosmia (normal olfaction) is classified as nIGD while those with olfactory deficits (anosmia or hyposmia) are diagnosed as KS [29].

Genetics studies coupled with animal models and advances in scientific tools have permitted identification of key genes in the pathogenesis of CHH and disruption of the HPG axis development. Synergistic effects of different mutations can explain the phenotypic variabilities of patients diagnosed with CHH. They have also revealed oligogenic inheritance as individuals can share genetic mutations and appear normal [30-32]. As of now, there are more than 60 loci identified for CHH and 17 have been linked to KS [27, 28, 33-34]. Table 1 contains a list of the most common (~ 5% or more) genes associated with KS.

Table 1.1: Most common genetic causes in IGD patients. *Modified from Balasubramanian and Crowley, 2022* [29].

Gene	Name	% of IGD attributed to pathogenic genetic variants
NOS1 (*KAL1)*	Anosmin 1	5%-10% (KS)
CHD7	Chromodomain-helicase-DNA-binding protein 7	5%-10% (KS or nIGD)
FGFR1	Fibroblast growth factor receptor 1	~10% (KS or nIGD)

Gene	Name	% of IGD attributed to pathogenic genetic variants
PROKR2	**Prokineticin receptor 2**	**~5% (KS or nIGD)**

1.2 Kallmann Syndrome

The earliest characterization of congenital atrophy of the testicles with lack of olfactory nerves was documented by Spanish scientist Aureliano Maestre de San Juan in 1856 [35]. Eighty-eight years later, Franz Josef Kallmann outlined the hereditary aspects of hypogonadism with anosmia by studying three affected families [36]. Hypogonadism and absence or impaired olfactory bulb development continued to be documented in humans until the seminal study of Naftolin and colleagues (1971) describing that GnRH hormone deficiency was the cause of infertility in KS patients [37].

Among the genetic causes of KS, several genes have been identified and found in families affected by this clinical condition.

1.2.1 ANOS1/KAL1

The gene ANOS1/*KAL1* is located on chromosome Xp22.31. It is composed of 14 exons that encode the protein anosmin-1, an extracellular matrix glycoprotein of 100kDa. It was the first protein discovered to be involved in GnRH neuronal migration [38]. Anosmin 1 is a cysteine rich protein with four fibronectin type III domains that interacts with components of the extracellular matrix. It appears to stimulate afferent projections to the olfactory bulb therefore essential for

guidance and migration of GnRH neurons [39-40]. This gene follows an X-linked inheritance patterns and has been reported in 5-10% of KS patients [39, 41-42].

1.2.2 FGFR1

FGFR1 gene, also called *KAL2*, is located on chromosome 8p11.23. It is composed of 18 axons that encode fibroblast growth factor receptor 1. This gene is alternatively spliced generating multiple splice variants and is a member of the receptor tyrosine kinase [43]. *FGFR1* expression is found in the nasal epithelium and in the vomeronasal organ and is required for olfactory bulb morphogenesis [44-45]. FGFR1 signaling pathway is amplified by the modulatory co-ligand, anosmin 1, inducing neurite outgrowth in human embryonic GnRH olfactory neuroblasts [46]. FGFR1 signaling and GnRH neuronal fate was also investigated in mice confirming its role in GnRH development [47]. Loss of function mutation in FGFR1 is responsible for autosomal dominant KS [48]. Mutations in this gene can account for an estimated 10% of KS or nIGD patients [29,49].

1.2.3 CHD7

CHD7 is located on chromosome 8q12.2. It is composed of 38 exons and codes for the chromodomain helicase DNA-binding protein 7 (CHD7). Over half of patients diagnosed with CHARGE syndrome, a disordered characterized by eye coloboma, heart defects, choanal atresia, retardation of growth and development, gonadal defects, and ear/hearing abnormalities, harbor mutations in this gene [50]. *CHD7* mRNA expression has been reported in migratory and post migratory GnRH neuronal cell lines. Additionally, expression has been reported in the hypothalamus, pituitary, and olfactory bulb of rats [51]. CHD7 has also been shown to regulate neural crest cell guidance genes as well as *SEMA3A*. SEMA3A is important for olfactory system

development and GnRH neuronal migration [52-55]. Mutations in this gene are found in 5-10% of cases with nIGD [29, 51, 56-57].

1.2.4 PROKR2

PROKR2 is located on chromosome 20p12.3. The gene encodes the prokineticin receptor 2, a G-protein coupled receptor belonging to the rhodopsin family [58-60]. PROKR2 is a 7-transmembrane domain protein with an extracellular N-terminus and an intracellular C-terminus. Engagement of PROKR2 by its ligand, prokineticin receptor 2, results in conformational changes that can promote intracellular Ca^{++} mobilization (activates Gαq/11 proteins and the intracellular effector phospholipase C) and are also capable of coupling and activating Gi, Gs pathways (Gαs proteins which activates adenylyl cyclase) [58-59,61]. Experiments using knockout mice for PROKR2 have determined that PROKR2 signaling is critical for olfactory bulb morphogenesis and sexual maturation and loss-of-function mutations in PROKR2 results in impaired GnRH neuronal migration [30, 62-63,71]. An estimated 5-10% of IGD patients harbor mutations in PROKR2 [29,49].

1.3 Prokineticins Role in Reproduction and Kallmann Syndrome

Prokineticins have been studied in a wide range of physiological functions across species. Prokineticin 1 (PROK1) and Prokineticin 2 (PROK2) are closely related neuropeptides initially characterized as proteins involved in generating contractions in the smooth muscle of the gastrointestinal tract [64]. Subsequently, research from different laboratories has implicated prokineticin signaling in regulation of circadian rhythms, metabolism, pain perception, immune responses and haematopoeisis [65-70]. Research focused on the brain and olfactory system

showed that prokineticin signaling has also a role in reproductive function, and olfactory bulb development [62, 71-72].

1.3.1 Ligands: PROK1 and PROK2

The gene for PROK1 is located on chromosome 1p13.3 in humans while the gene for PROK2 is located on chromosome 3p21.1. These ligands share an estimated 44% amino acid sequence. PROK1 is an 86 amino acid protein while PROK2 is an 81 amino acid protein. They share the N-terminal sequence AVITGA, that has been deemed important for their biological function, and conserved cysteines believed to form disulfide bonds [60,73]. Genetic mutations in this conserved region results in modifications of the cysteine residues and no biological activity [73]. PROK1 and PROK2 are for the most part differentially expressed in tissues. PROK1 expression has been found predominantly in the ovary, testis, adrenal cortex and placenta [74, 58, 75-77] and PROK2 has high expression in the central nervous system [65, 78-81].

PROK1 and PROK2 share affinity to two G-protein coupled receptors, prokineticin receptor 1 and 2 (PROKR1 and PROKR2) [58, 61, 82]. Activity and function of both ligands is limited by their differential expression, but also the expression patterns of the target receptors.

1.3.2 Receptors: PROKR1 and PROKR2

PROKR1 and PROKR2 show an estimated 87% amino acid sequence similarity [61, 64, 82]. Despite their similar biochemical properties, PROKR1 and PROKR2 receptors are differentially expressed and have been linked to different physiological functions. PROKR1 is mainly expressed in peripheral tissues including the gastrointestinal tract, whereas PROKR2 is primarily

expressed in the brain and olfactory structures [61,64, 79, 83]. Both ligands, PROK1 and PROK2 can activate PROKR2 with similar potency [82].

1.3.3 PROK2/PROKR2 in Reproduction

PROKR2 has been implicated in the regulation of reproduction in both humans and mice. PROKR2 was identified as a novel gene in KS in a cohort of 192 patients. It was found that patients in this study harbored heterozygous, homozygous, or compound heterozygous mutations in the *PROKR2* gene [84]. Individuals harboring PROKR2 mutations had different degrees of reproductive dysfunction and olfactory deficits [84]. A second study further reported the variability in reproductive dysfunction in individuals harboring mutations in the PROK2/PROKR2 system. A homozygous deletion in the PROK2 gene, resulting in a protein lacking bioactivity, was identified in two siblings with varying IGD. Only one of them had anosmia. Another sibling harboring a heterozygous mutation was unaffected [30]. Further mutations in *PROK2* and *PROKR2* genes have been reported, highlighting the complexities of *PROK2/PROKR2* in the pathogenesis of KS [85-93]

Functional studies focused on *PROK2/PROKR2* mutations were boosted by the development of the *Prokr2* null mice. These mice display a KS-like syndrome with atrophy of the reproductive system and hypoplasia of the olfactory bulbs [71]. These findings were unique to *Prokr2* null mice because despite the reported similarity in sequence with *Prokr1, Prokr1* null mice had no reproductive or olfactory deficits. *Prokr2* null mice had reduced number of GnRH neurons in the medial septum and preoptic area and decreased GnRH innervation of the median eminence, suggesting impairment in GnRH neuronal migration [71]. Similarly, *Prok2* null mice showed impaired olfactory bulb morphogenesis, neurogenesis and also had reproductive deficits [30, 62].

Together, these studies demonstrate that PROK2-induced PROKR2 signaling is required for olfactory bulb development, GnRH neuronal migration and sexual maturation. It is worth noting that immunohistochemistry analysis has shown that GnRH neurons do not express detectable PROKR2 and therefore they are likely two distinct populations interacting via an unknown mechanism [30]. In both studies, heterozygous mice were not significantly affected.

The *Prokr2* null mice uncovered a reproductive role of this system, particularly the GnRH neuronal migration. These mouse models, however, prevent further studies of the role of PROKR2 in reproduction since they lack adequate GnRH neuronal migration and proper reproductive neuroendocrine function.

1.4 Current Mouse Models for PROK2/PROKR2 studies

Limitations in animal models have hindered research in physiological regulated by prokineticins. Several transgenic mice have been created to study aspects of PROK2/PROKR2. Besides previously mentioned animal models, there are a few others that have been used to understand the PROK2/PROKR2 system. For example, a PROK2-GFP mouse model was used to assess estrogen receptor colocalization [72]. Mice with deletion or overexpression of *Prok2* and PROKR2-GFP mice were produced to assess PROK2 role in the suprachiasmatic circadian clock [95- 97]. It is believed that PROK2 is an output molecule for the circadian clock. Altogether, these mouse models serve as method to label and characterize these neurons and identify coexpression of neurotransmitters, transcription factors, receptors, and other peptides. The limitation of these mouse models is the impossibility of manipulating subpopulations of

PROKR2 expressing neurons, and the inability to study the PROKR2 system past development without the confounding variable of neuronal migration and developmental compensation.

With advances in technology our lab was able to generate a mouse model with Cre recombinase expression driven by *Prokr2* gene. With this mouse model, we identified several regions in the brain that display sexually dimorphic expression of PROKR2 [81]. This mouse model allows for the implementation of techniques for remote control of neuronal signaling on populations expressing *Prokr2.* Additionally, it allows for defining brain circuitry applying Cre-induced molecular tracers, deleting genes within PROKR2 expressing cells and characterizing the physiological effects of this deletion. Most importantly, it allows for targeting specific reproductive control sites to further understand the PROKR2 system in adult reproduction.

1.5 Current Gap in Knowledge

Despite the knowledge of expression profiles of PROKR2 and its ligands, the role of Prokr2 in specific brain sites remains unknown. The models prior to our ProkR2-Cre mice, did not allow for the assessment of PROKR2 role in adult reproduction. This is particularly relevant because studies have suggested that the PROKR2 heterozygous null mice displayed abnormal estrous cycles under normal GnRH neuronal migration. It is currently unknown which population of PROKR2 expressing neurons respond to changes in gonadal steroids milieu, where they project and what is the role of distinct subpopulations of PROKR2 expressing cells. Functional roles of specific PROKR2 expressing neurons in reproduction remains to be elucidated.

1.5.1 Hypothesis

We *hypothesize* that ProkR2 expressing neurons have a role in adult reproductive function. The *objective* of my doctoral studies was to investigate if PROKR2 has a role in reproductive function beyond GnRH migration. My findings are subdivided in the following chapters:

In chapter 2, I describe the ProkR2-Cre neurons that co-express gonadal steroid receptors relevant for reproductive success: the estrogen receptor alpha (ERα) and the androgen receptor (AR). I show that the only brain site co-expressing Prokr2 and either receptor is the posterior amygdala. This chapter was published in Frontiers in Neuroanatomy [98].

In chapter 3, I describe the projection pattern of ProkR2-Cre neurons in the posterior amygdala of male and female mice using an adeno-associated viral vector (AAV) for molecular mapping.

In chapter 4, I performed a series of functional studies (i.e., sexual behavior, response to opposite and same sex odors, chemogenetic activation and inhibition) to uncover the role of ProkR2 in the posterior amygdala. I show that these neurons are highly responsive to opposite sex odor and have a role in opposite sex recognition in males.

In chapter 5, I discuss the findings in an integrated format, the limitations of my studies and propose future directions for the field.

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Chapter 2 Sex Differences in the Coexpression of Prokineticin Receptor 2 and Gonadal Steroids Receptors in Mice

2.1 Abstract

Loss-of-function mutations in prokineticin 2 (*PROK2*) and the cognate receptor prokineticin receptor 2 (*PROKR2*) genes have been implicated in reproductive deficits characteristic of Kallmann Syndrome (KS). Knock out of *Prokr2* gene produces the KS-like phenotype in mice resulting in impaired migration of gonadotropin releasing hormone (GnRH) neurons, olfactory bulb dysgenesis and infertility. Beyond a developmental role, pharmacological and genetic studies have implicated PROKR2 in the control of estrous cycle in mice. However, PROKR2 is expressed in several reproductive control sites but the brain nuclei associated with reproductive control in adult mice have not been defined. We set out to determine if ProkR2 neurons in both male and female mouse brains directly sense changes in gonadal steroids milieu. We focused on estrogen receptor α (ERα) and androgen receptor (AR) due to their well-described function in reproductive control via actions in the brain. We found that the ProkR2-Cre neurons in the posterior nucleus of the amygdala has the highest colocalization with ERα and AR in a sex-specific manner. Few colocalization was found in the lateral septum and in the bed nucleus of the stria terminalis, and virtually no colocalization was observed in the medial amygdala. Our findings indicate that the posterior nucleus of the amygdala is the main site where PROKR2 neurons may regulate aspects of the reproductive function and social behavior in adult mice.

2.2 Introduction

A spectrum of reproductive deficits and anosmia, or loss of smell, are characteristics of patients diagnosed with Kallmann Syndrome (KS). Complex genetic studies in humans as well as data from mouse models have identified several genes associated with this clinical condition [1]. Among them, a series of distinct mutations in the prokineticin receptor 2 (*PROKR2*) gene have been identified in KS patients [2-4]. *Prokr2* knockout mice reproduce the phenotype of patients with KS, as they show severe defects in olfactory bulb development, a significant decrease in numbers of gonadotropin releasing hormone (GnRH) neurons in the medial septum-preoptic area, and a decrease in GnRH fibers in the median eminence [5-6]

While the loss of function mutation in both *Prokr2* alleles results in impaired GnRH neuronal migration, *Prokr2*-null heterozygous mice show regular number of GnRH neurons within the hypothalamus suggesting normal migration. Interestingly, these mice exhibit disrupted estrous cycle indicating that, despite normal GnRH migration, the reproductive function is compromised [7]. However, the brain sites associated with the role of PROKR2 neurons in adult reproduction is not known.

Initial studies in male mice have shown that *Prokr2* mRNA is expressed in several reproductive control sites, including the lateral septum, preoptic area, and arcuate nucleus, and strong expression in the medial nucleus of the amygdala [8]. Neurons of the lateral septum, preoptic area and medial amygdala integrate olfactory stimuli and mediate the appropriate reproductive endocrine and behavioral responses [9-11].

With the aim of increasing the experimental tools to assess the role of PROKR2 in adult reproduction, we developed a mouse model expressing Cre recombinase driven by the *Prokr2* promoter [12]. Using the ProkR2-Cre reporter mouse, we reproduced the previous findings in males and expanded the data in females [12]. These findings outlined the distribution of neuronal *Prokr2* and putative roles in reproductive physiology and sexual behavior guided by prokineticin actions in the brain. Although the expression profile of *Prokr2* in male and female brains have been characterized, it is not clear if subpopulations of neurons sense changing levels of circulating gonadal steroids required for estrous cycle and social behavior.

Therefore, to begin assessing potential brain sites where PROKR2 cells influence the reproductive axis, we performed a systematic evaluation of the coexpression of estrogen receptor-α (ERα) and androgen receptor (AR) with ProkR2-Cre neurons in male and female brains using the ProkR2-Cre mouse model [12]. We focused on ERα and AR due to their well-described role in adult reproduction in both sexes [13-26].

2.3 Materials and Methods

2.3.1 Animals

Adult (2-5 months old) male and female B6;SJL-*Prokr2*$^{em1(cre)Cfe}$ (ProkR2-Cre mice) were used for all experiments (JAX® stock #043846-JAX , [12]). The ProkR2-Cre mice were crossed with Cre-dependent, eGFP-L10a mice kindly provided by Dr. David Olson at the University of Michigan and commercially available (JAX® stock # 024750, [27]). All mice were

heterozygous for the ProkR2-Cre allele with one or two copies of the eGFP-L10a allele. Mice were maintained in a light- (12L:12D) and temperature- (21-23°C) controlled environment at the University of Michigan Animal Facility (ULAM). Animals had free access to water and a phytoestrogen-reduced Envigo diet 2016 (16% protein/4% fat). A phytoestrogen-reduced diet was used to minimize effects of exogenous estrogen in the expression of gonadal steroids receptors [28-31]. All procedures and experiments were done in accordance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan (protocol # PRO00010420).

All mice were genotyped as outlined in Mohsen et al., 2017 and Krashes et al., 2014 [12, 27].

2.3.2 Perfusion and brain histology

Mice were anesthetized with isoflurane (Fluriso; Vet One) and transcardially perfused with 0.1M PBS followed by 10% neutral buffered formalin. All perfusions were done between 12:00 to 2:00PM (corresponding to ZT6 and ZT8). Following perfusion, brains were collected and postfixed in 20% sucrose-10% formalin for 2 h. Brains were cryoprotected in 20% sucrose-PBS and sectioned in freezing microtome (Leica). Four series of 25-µm thick coronal sections were collected and stored at -20°C in cryoprotectant.

2.3.3 Dual label immunoperoxidase

Sections were washed with 0.1M PBS to remove cryoprotectant and incubated in 0.3% hydrogen peroxide (H_2O_2) in Triton-PBS (0.25%) for 30 min. Sections were then washed in 0.1M PBS and were incubated in primary antibody. The following antibodies and concentrations were used: rabbit anti-estrogen receptor-α (ERα) primary antibody (1:20,000, Millipore Cat# 06-935, RRID: AB_310305) and rabbit anti-androgen receptor (AR) antibody (1:400, Abcam Cat# ab133273, RRID: AB_11156085). All primary incubation were prepared with 3% donkey serum in Triton X100-0.1M PBS (0.25%) and tissues were incubated overnight at room temperature. The following day, sections were washed in 0.1M PBS and incubated in secondary biotin-conjugated donkey anti-rabbit antibody for 1 h (1:1,000, Jackson ImmunoResearch Laboratories), washed again in 0.1M PBS and incubated in Avidin-Biotin Complex (ABC) in 0.1M PBS (1:500, Vector Labs) for 1 h. The peroxidase reaction was performed using 0.05% 3,3'-diaminobenzidine (DAB, Sigma) and 0.05% nickel ammonium sulfate (Nickel, Sigma) as chromogens and 0.01% hydrogen peroxidase. Following 0.1M PBS washes and another 30 min 0.3% hydrogen peroxide (H_2O_2) incubation, sections were incubated in chicken anti-GFP primary antibody (1:20,000, AvesLabs, catalog #GFP-1010; RRID: AB_2307313) with 3% donkey serum in Triton-0.1M PBS (0.25%). The following day, the same steps previously described were followed except that a secondary biotin-conjugated donkey anti-chicken antibody (1:1,000, Jackson ImmunoResearch Laboratories) was used and Nickel was omitted from the DAB solution. Sections were mounted on gelatin coated slides, dehydrated, delipidated in xylenes for 15 min and coverslipped with DPX (EMS, Hatfield, PA).

Series of brain sections were counterstained with thionin for anatomical references. Slides were dipped in 0.25% thionin for 1 min, dehydrated, delipidated in xylenes for 15 min and coverslipped with DPX (EMS, Hatfield, PA).

2.3.4 Imaging and data analysis

Brain regions were evaluated using the digital Allen Mouse Brain atlas and sites showing colocalization of AR or ERα with ProkR2-Cre eGFP-L10a positive cells were identified. Quantification of dual labeled neurons was performed using ImageJ cell counter plug-in and Graphpad was used for data analysis. Only cells showing clear nuclear staining were deemed as dual labeled. Subjective analysis comparing dual labeled neurons based on relative expression (e.g., no dual labeled neurons visualized= - ; highest dual labeled neurons visualized=+++) was performed by two independent evaluators. To determine potential sex differences in number of neurons in the PA, we quantified the most posterior level of the PA (where we see highest colocalization of ProkR2-Cre eGFP and AR or ERα) using thionin staining. Only one section and one side of the amygdala was quantified/mice. All blue neurons and dark brown (blue + eGFP labeled) were quantified. Microphotographs were acquired using the upright microscope Zeiss Axio Imager M2 and a digital camera (Axiocam, Zeiss) using the Zen software. Images were organized in figures using Adobe Photoshop. Brightness, sharpness and contrast were adjusted for image presentation.

2.3.5 Statistics

Analysis was performed using Prism, version 9 (GraphPad Software Inc). Data are reported as the mean ± SEM. Normal distribution of data was determined using a Shapiro-Wilk test

(significance alpha 0.05). An unpaired *t* test with Welch's correction was used to analyze data. $P<0.05$ was considered statistically significant. Data are reported as the mean ± SEM.

2.4 Results

We performed a systematic evaluation of the ProkR2-Cre L10 eGFP neurons that co-express AR or ERα in male and female brains (Table 1, n=5 males for AR and ERα, n=3 females for AR and n=6 females for ERα). Because levels of estradiol may alter *Esr1* expression in opposite ways in different nuclei, we used cycling females (undefined estrous cycle stage) to avoid the effect of excess (E2 treatment) or lack (ovariectomy) of estradiol in specific nuclei [32]. We focused our analysis on the forebrain where virtually all reproductive control sites are located.

2.4.1 Distribution of AR and ERα in ProkR2-Cre eGFP neurons of male mice.

The analysis of colocalization of AR or ERα with ProkR2-Cre eGFP was focused on forebrain nuclei previously described to express *Prokr2* mRNA and the reporter gene [8,12]. The lateral septum and the bed nucleus of the stria terminalis had minimal colocalization of ProkR2-Cre eGFP and AR (Fig. 2.1, A-D). Virtually no colocalization of Prokr2 GFP with AR was found in the suprachiasmatic nucleus (Table 1). We also found minimal colocalization in the medial preoptic nucleus (Fig. 2.1, E-F), paraventricular nucleus of the hypothalamus (Table 1), arcuate nucleus (Fig. 2.1, G-H) and ventral premammillary nucleus (Table 1). No colocation of ProkR2-Cre eGFP and AR was observed in the ventromedial hypothalamic nucleus (Fig. 2.1, I-J). In the amygdala, virtually no colocalization was observed in the posterodorsal subdivision of the medial nucleus (Fig. 2.1, K-L). However, high numbers of ProkR2-Cre eGFP immunoreactive

cells of the posterior amygdala (PA, a.k.a., amygdalohypothalamic area or AHi) coexpress AR (Table 1 and Table 2).

Similarly, virtually no colocalization between ProkR2-Cre eGFP and ERα was found in the lateral septum, bed nucleus of the stria terminalis, medial preoptic area, arcuate nucleus, ventromedial hypothalamic nucleus, and posterodorsal subdivision of the medial amygdala (Fig. 2.2, A-L). Moderate colocalization with ProkR2-Cre eGFP was also observed in the PA (Table 1 and Table 2).

2.4.2 Distribution of AR and ERα in ProkR2-Cre eGFP neurons of female mice.

Female brains showed a comparable coexpression pattern to male brains. The lateral septum had minimal colocalization between ProkR2-Cre eGFP and AR, while sporadic colocalization was observed in the bed nucleus of stria terminalis (Fig. 2.3. A-D). No colocalization between ProkR2-Cre eGFP and AR was found in the suprachiasmatic nucleus, and minimal colocalization was observed in the medial preoptic nucleus, paraventricular hypothalamus, and arcuate nucleus (Fig. 2.3. E-H, Table 1). Very few ProkR2-Cre eGFP cells colocalize AR in the ventromedial hypothalamus (Fig. 2.3, I-J). We found virtually no colocalization of ProkR2-Cre eGFP and AR in the posterodorsal subdivision of the medial medial amygdala (Fig. 2.3., K-L). Small coexpression of ProkR2-Cre eGFP and AR was observed in female PA (Table 1 and Table 2).

Colocalization of ProkR2-Cre eGFP with ERα was briefly reported by our lab [12]. In a more comprehensive analysis, in this study we reproduced our initial findings and found no colocalization of ProkR2-Cre eGFP and ERα in the bed nucleus of the stria terminalis and

arcuate nucleus (Table 1), and very low colocalization in the ventromedial nucleus of the hypothalamus (Table 1). In addition, we found very low colocalization of ProkR2-Cre eGFP and ERα in the paraventricular nucleus of the hypothalamus (Table 1). Higher colocalization was observed in the PA (Table 1 and Table 2).

Table 2.1: Subjective analysis of the coexpression of ProkR2-Cre eGFP and androgen receptor (AR) or estrogen receptor α (ERα) immunoreactivity in male and female brains. Only Notations -, +/−, +, and +++ represent absent, very low, low, and high colocalization. The Allen Mouse Brain atlas was used as a reference for brain nuclei and localization (atlas image).

Brain areas and nuclei	**Atlas Image**	**Male**		**Female**	
		AR	**ERα**	**AR**	**ERα**
Lateral septal nucleus, rostral (LSr)	53	+	-	+	+/-
Medial preoptic nucleus (MPN)	55	+	-	+/-	+/-
Bed nucleus of stria terminalis, posterior (BSTp)	56	+/-	-	+/-	+/-
Suprachiasmatic nucleus (SCH)	59	-	-	+/-	-
Paraventricular nucleus of hypothalamus (PVH)	61	+	+/-	+/-	+
Arcuate nucleus (ARH)	69	-	-	+/-	+/-
Medial nucleus of the amygdala, posterodorsal (MEApd)	70	-	-	-	-
Ventromedial nucleus, ventrolateral (VMHvl)	70	-	-	+	+
Ventral premammillary nucleus (PMv)	76	-	-	+/-	-
Posterior nucleus of the amygdala (PA)	80	+++	+++	+	+++

2.4.3 Colocalization of ProkrR2-Cre eGFP with AR or with ERα in the posterior nucleus of the amygdala (PA) shows sex differences.

The PA is the forebrain site with highest colocalization of ProkR2-Cre eGFP and AR or ERα. Because in previous studies we found sex differences in ProkR2-Cre eGFP expression [12], we performed a careful quantification of colocalization to compare the findings between sexes (Table 2).

In males, about 24.7 ± 6.8% of ProkR2-Cre eGFP cells expressed AR (Fig. 2.4, A-B, Table 2), whereas 70.6 ± 7.5% of AR cells were ProkR2-Cre eGFP positive. In contrast, female brains had much lower colocalization (Fig. 2.4, C-D, Table 2). About 4.5 ± 1.8% of ProkR2-Cre eGFP cells expressed AR and 22.3 ± 4.3% of AR cells coexpressed ProkR2-Cre GFP.

Similar coexpression between ProkR2-Cre eGFP and ERα was observed in males and females PA (Fig. 2.4 E-H, Table 2). About 21.8 ±2.6% of ProkR2-Cre eGFP cells in males coexpressed ERα while 23 ± 2.5% of ProkR2-Cre eGFP cells in females coexpressed ERα. Of note, about 70% of ERα in males where ProkR2-Cre eGFP cells, while about 50% of ERα-ir neurons coexpress ProkR2-Cre eGFP in the female PA (Table 2).

To assess if the PA of males and females shows different number of neurons, we quantified total number of cells using thionin staining at the level colocalizations were reported (Allen Brain Atlas, level 80, N=3, male and female). No differences were observed. We found that males had on average 463.67 ± 11.7 cells and females 491.33 ± 45.41 in the PA ($P > 0.05$, Fig. 2.5, A-D).

Table 2.2: Quantification of cells coexpressing ProkR2-Cre eGFP and androgen receptor (AR) or estrogen receptor α (ERα) immunoreactivity in the posterior nucleus of the amygdala of male and female mouse brains.

AR	**Total GFP-ir**	**Total AR-ir**	**% Dual labeled /Total GFP-ir**	**% Dual labeled/ Total AR-ir**
Male	323.4 ± 17.57	111 ± 32.88	24.7 ± 6.8%	70.6 ± 7.5%
Female	176 ± 23.29	33.33 ± 11.62	4.5 ± 1.8%	22.3 ± 4.3%
ERα	**Total GFP-ir**	**Total ERα-ir**	**% Dual labeled /Total GFP-ir**	**% Dual labeled/ Total ERα-ir**
Male	333.2 ± 28.5	100.2 ± 10.39	21.8 ± 2.6%	71.8 ± 5.5%
Female	193.67 ± 12.93	89.67 ± 15.28	23.0 ± 3.5%	53.8 ± 7.4%

2.5 Discussion

In the present study, we performed a systematic evaluation of the colocalization of ProkR2-Cre eGFP with AR or ERα immunoreactivity in the forebrain of male and female mice. Previous work from our and other laboratories have characterized the expression of AR in rodents [17-18,33-36]. AR is highly expressed in the lateral septum, bed nucleus of the stria terminalis, medial and posterior nucleus of the amygdala, and several nuclei of the hypothalamus, including the medial preoptic, the suprachiasmatic, the paraventricular, the arcuate and the ventral premammillary nucleus AR expression in the female brain is observed in nearly the same sites as male brains, albeit in much lower density [35,37]. ERα expression has been previously described to be dense in the lateral septum, bed nucleus of the stria terminals, the medial preoptic nucleus, the arcuate nucleus, the medial amygdala, and the posterior nucleus of the amygdala among

several other hypothalamic nuclei of both sexes [18, 33,37-40. Interestingly, most of the sites expressing these sex steroid receptors are also sites that express *Prokr2* [8,12].

Having verified that our immunohistochemistry is consisted with previous reports, we found that out of all forebrain sites expressing ProkR2-Cre, the highest colocalization with AR and ERα was observed in the PA of both sexes. We found, however, a clear sex difference with males showing high rates of ProkR2-Cre eGFP neurons coexpressing AR or ERα. These findings place the PA as the main site by which the *Prokr2* neuronal circuit sense and may respond to changes in circulating levels of gonadal steroids potentially modulating reproductive physiology and/or sex and social behaviors. To date, studies investigating the relevance of PROKR2 in reproductive physiology and behaviors have been scarce. The *Prokr2* homozygous null mice exhibited deficits in GnRH neuronal migration and reproductive organs development. The heterozygous null mice, however, were not different from wildtype littermates [6]. The fertility of the *Prokr2* null heterozygous mice was not assessed, given the normal migration of GnRH neurons and no behavioral assays were performed in neither female nor male mice. Those heterozygous null mice however exhibited irregular estrous cycles, but the *Prokr2* neural circuitry associated with this observation remains unknown.

The PA is an understudied site as limited publications have focused on the circuitry, chemical phenotype, and functionality of its neurons. Most of the literature on amygdala's role in sex and social behaviors has focused on the subdivisions of the medial amygdala. Extensive literature looking at the effects of lesion of the medial amygdala, the projections to and from the medial amygdala, have shown that it plays a critical role in sexual behaviors and aggression, mating and

associated behaviors, and it is critical in processing olfactory cues [10, 22,41-51. Few studies have also suggested that the PA is associated with chemosensory pathways related to reproductive behaviors. For instance, studies in Syrian hamsters described that the PA expresses Fos-immunoreactivity, a marker of neuronal activation, in both mating and agonistic behaviors [52-53] .

Using retrograde and anterograde tracers in rats, studies revealed that innervation of the PA comes from areas associated with processing odor inputs from the vomeronasal system, such as the posterodorsal subdivision of medial amygdala [54]. The PA also innervates areas such as the lateral septum, and the bed nucleus of the stria terminalis [54]. These findings suggested that the PA is relevant for social behaviors and, together with the medial amygdala, comprises the "olfactory amygdala" [43, 54-55].

The murine PA expresses a dense collection of gonadal steroids receptor [18, 33,35-39]. Specifically, the PA neurons express AR and ERα, and about half of steroid receptor containing neurons expresses both [18]. In rats, the sites innervated by the PA are also dense in gonadal steroids receptors further implicating this nucleus in neural circuitry sensing circulating gonadal steroids [33, 56].

We showed previously that a higher number of *Prokr2* and ProkR2-Cre eGFP expressing cells is observed in the PA of males [12]. This finding invited speculation on the potential role of PA PROKR2 neurons in both sexes. That ProkR2-Cre eGFP cells of the PA of male and female mice

coexpress gonadal steroid receptors further emphasize a reproductive relevant role for these neurons. Sex differences and the role of gonadal steroids receptors in a specific subpopulation of PA PROKR2 expressing cells need further investigation.

Expression of the ligand, prokineticin 2, has been characterized in mice and in the macaque monkey brain [8, 57]. Expression patterns were similar in both species. The areas known to highly express prokineticin 2 relevant to reproduction are the olfactory bulb, the medial amygdala, the medial preoptic area, the arcuate nucleus, and the suprachiasmatic nucleus [8]. Whether prokineticin 2 neurons in these or alternative brain sites project to PA is not known. Additional studies are needed to inform which prokineticin 2 expressing sites innervate the PA.

Use of genetic manipulations of PA neurons including remote control (i.e., opto or chemogenetic) is very limited but in recent years, a study in male mice showed that a subpopulation of these neurons coexpresses vesicular glutamate transporter 1 (Vglut1). The PA Vglut1 neurons project to the ventrolateral subdivision of the ventromedial nucleus of the hypothalamus and induce aggression when activated [58]. Another study identified two subpopulations of ERα PA neurons, one that projects to the medial preoptic nucleus and another that projects to the ventromedial hypothalamus [59]. The first was activated during mating and was necessary and sufficient for sexual behaviors in males and the second was activated during intermale aggression [59]. Collectively, these studies indicate that aggression and mating behavior are controlled by a subset of ERα PA cells. In both studies, it is likely that PROKR2 expressing cells were also activated, given that *Prokr2* gene is enriched in the PA and that a

subpopulation of PROKR2 PA neurons express ERα. [12, 59]. Projection profiles and remote control of PROKR2 expressing cells need further investigation to define the contribution of PROKR2 expressing cells to these behaviors. In addition, the role of AR in PA PROKR2 expressing cells of males and females are unknown.

The relevance of PROKR2 system in the development of the reproductive axis, has been described in both in humans and mouse models [1,5]. While this study does not assess the function of PROKR2 within the PA, it begins to unravel the components required for a role in sexual and/or social behavior, i.e., the potential to sense changes in circulating gonadal steroids in conspecific opposite sex mates. Further studies aiming at defining the associated neural circuitry and specific action of PROKR2/AR and/or PROKR2/ERα in reproductive function in both sexes are warranted.

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2.7 Copyright

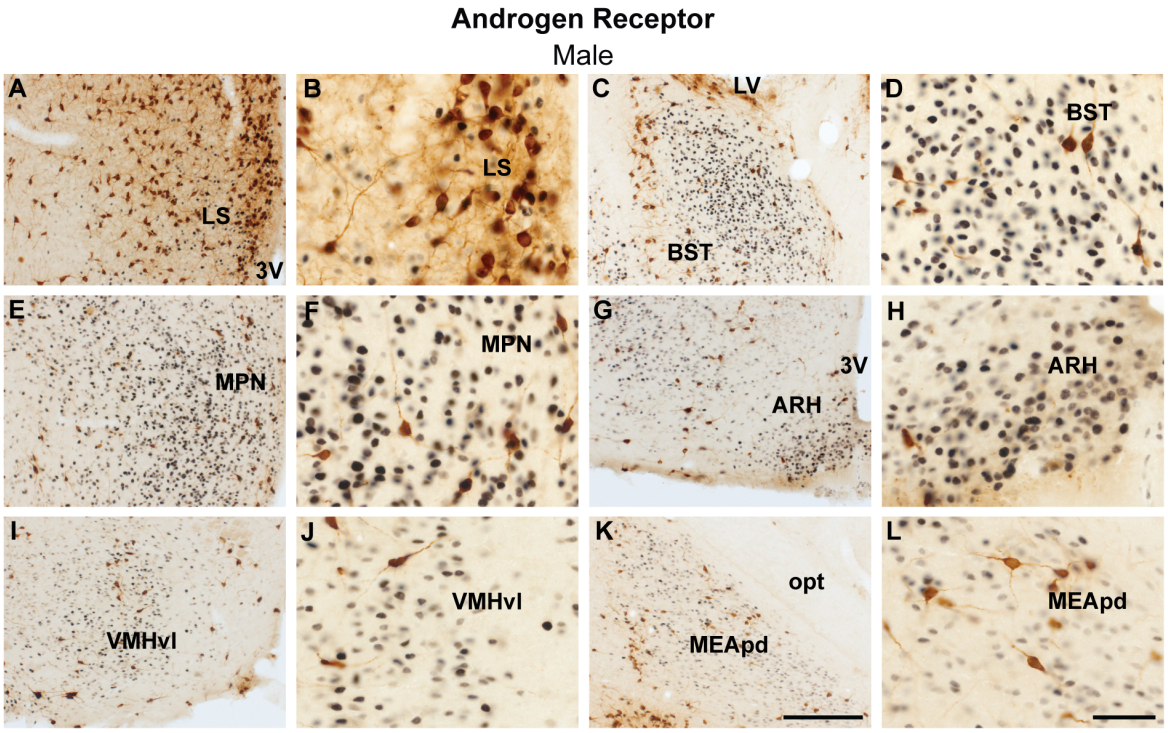

Figure 2.1: Coexpression of ProkR2-Cre eGFP and androgen receptor (AR) in the forebrain of the male mouse. (A–L) Bright field micrographs showing the expression of AR (black) and GFP (brown) immunoreactivity in the lateral septal nucleus, LS (A,B), the bed nucleus of stria terminalis, BST (C,D), the medial preoptic nucleus, MPN (E,F), the arcuate nucleus, ARH (G,H), the ventromedial hypothalamic nucleus, ventrolateral subdivision, VMHvl (I,J), the medial nucleus of the nucleus, and posterodorsal subdivision, MEApd (K,L). Abbreviations: LV, lateral ventricle; opt, optic tract; 3 V, third ventricle. Scale Bars: (A,C,E,G,I,K) = 200 µm; (B,D,F,H,J,L) = 50 µm.

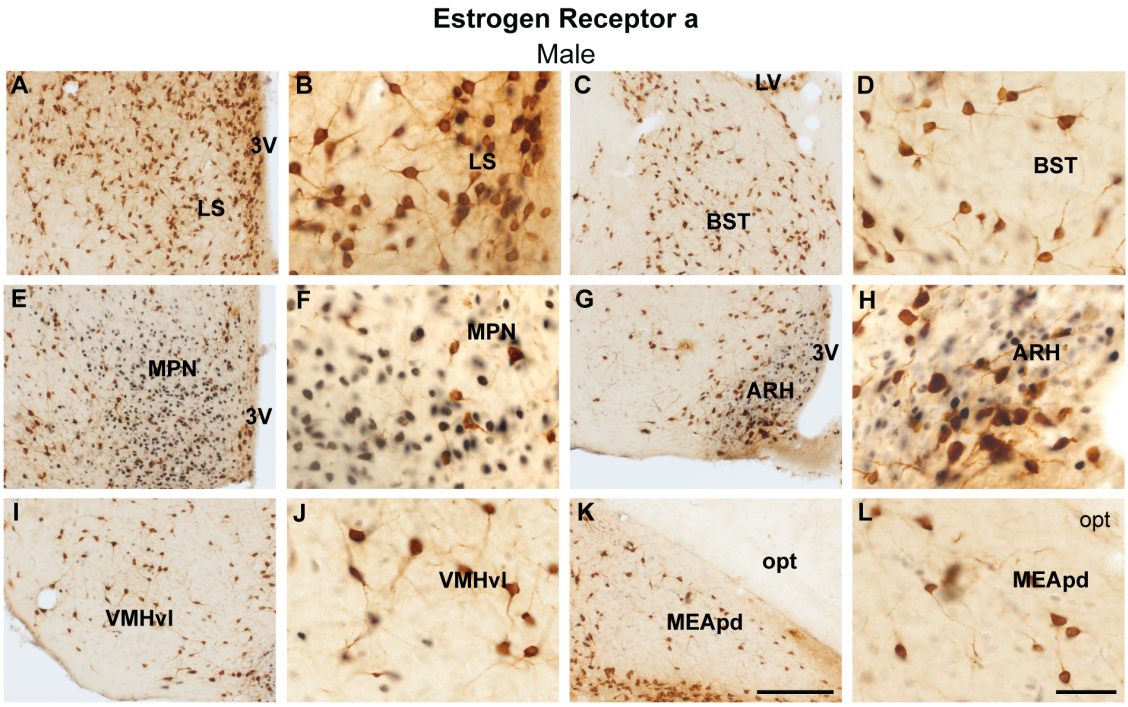

Figure 2.2: Coexpression of ProkR2-Cre eGFP and estrogen receptor α (ERα) in the forebrain of the male mouse. (A–L) Bright field micrographs showing the expression of ERα (black) and GFP (brown) immunoreactivity in the lateral septal nucleus, LS (A,B), the bed nucleus of stria terminalis, BST (C,D), the medial preoptic nucleus, MPN (E,F), the arcuate nucleus, ARH (G,H), the ventromedial hypothalamic nucleus, ventrolateral subdivision, VMHvl (I,J) and the medial nucleus of the amygdala, posterodorsal subdivision, MEApd (K,L). Abbreviations: LV, lateral ventricle; opt, optic tract; 3 V, third ventricle. Scale Bars: (A,C,E,G,I,K) = 200 μm; (B,D,F,H,J,L) = 50 μm.

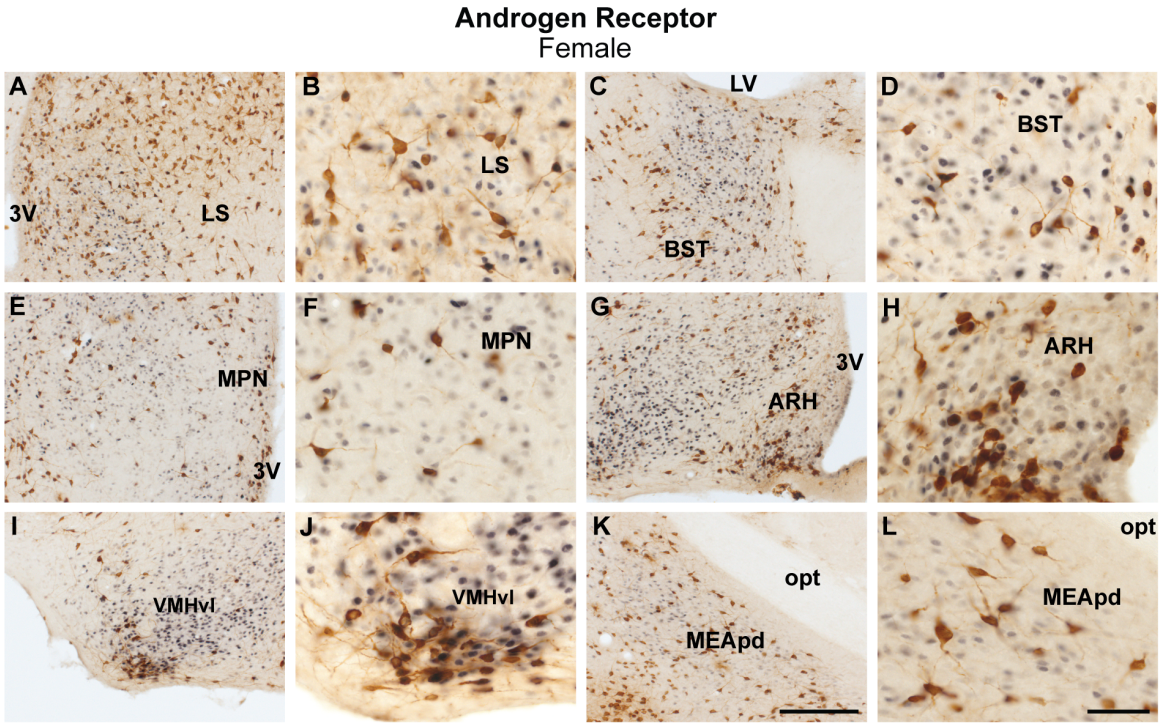

Figure 2.3: Coexpression of ProkR2-Cre eGFP and androgen receptor (AR) in the forebrain of the female mouse. (A–L) Bright field micrographs showing the expression of AR (black) and GFP (brown) immunoreactivity in the lateral septal nucleus, LS (A,B), the bed nucleus of stria terminalis, BST (C,D), the medial preoptic nucleus, MPN (E,F), the arcuate nucleus, ARH (G,H), the ventromedial hypothalamic nucleus, ventrolateral subdivision, VMHvl (I,J), and the medial nucleus of the amygdala, the posterodorsal subdivision, MEApd (K,L). Abbreviations: LV, lateral ventricle; opt, optic tract; 3 V, third ventricle. Scale Bars: (A,C,E,G,I,K) = 200 µm; (B,D,F,H,J,L) = 50 µm..

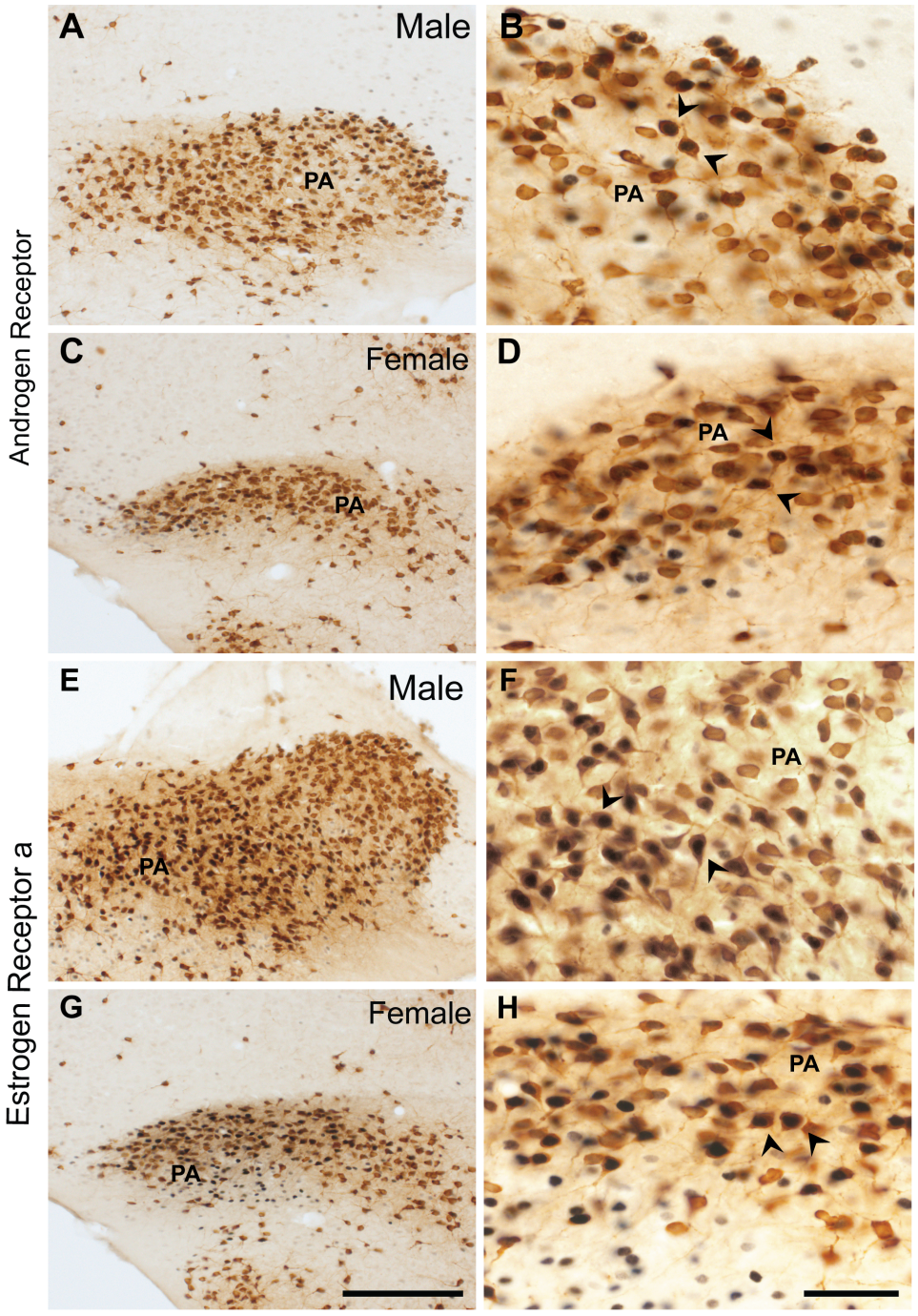

Figure 2.4: Coexpression of ProkR2-Cre eGFP with AR and in ProkR2-Cre eGFP with estrogen receptor α (ERα) in the posterior nucleus of the amygdala (PA). Bright field micrographs showing the expression of AR (black) and GFP (brown) immunoreactivity in the male PA (A,B) and in the female PA (C,D). Bright field micrographs showing the expression of ERα (black) and GFP (brown) in the male PA (E,F) and in the female PA (G,H). Arrows in (B,D,F, and H) indicate dual labeled neurons. Scale Bars: (A,C,E,G) = 200 µm; (B,D,F,H) = 50 µm.

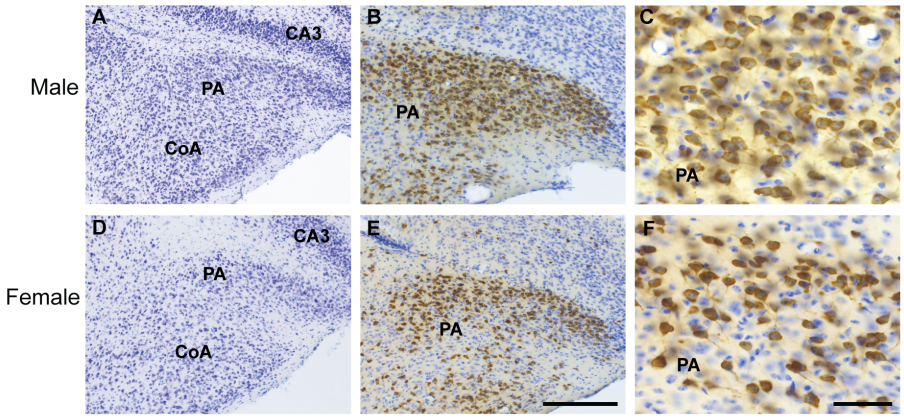

Figure 2.5: Cytoarchitecture and distribution of ProkR2-Cre eGFP neurons of the posterior amygdala (PA) of male and female brains. Brightfield micrographs showing expression of GFP (brown) and thionin positive cells (blue) in the male (A-C) and female PA (D-F). Abbreviations: CoA: Cortical amydala; CA3:field CA3 hippocampus Scale Bars: A-B, D-E = 200 μm; C, F = 50 μm.

Chapter 3 The Prokineticin Receptor 2 Neurons in the Posterior Amygdala Densely Innervate Forebrain Sites Associated with Reproductive Neuroendocrine Function and Motivated Behaviors

3.1 Introduction

The *Prokr2/PROKR2* gene is involved in regulating reproduction in both mice and humans. The *Prokr2* gene codes for prokineticin receptor 2, a G-protein coupled receptor highly expressed in the brain [1-3]. Studies in humans have identified several mutations in this gene linked to the genetic disorder Kallmann syndrome [4-9]. Kallmann syndrome is characterized by abnormal function of the reproductive axis and impaired sense of smell or anosmia [10-13]. Studies in mice have determined that the abnormal development of the reproductive axis is due to impaired migration of gonadotropin-releasing hormone (GnRH) neurons, which in turn result in no active release of sex steroids such as testosterone and estrogen. Further developmental and functional studies are needed to determine the PROKR2 function in GnRH neuronal migration.

We previously characterized the ProkR2-Cre mouse model and assessed the co-expression of sex steroid receptors, estrogen receptor α (ERα) and androgen receptor (AR) in ProkR2-Cre expressing neurons of male and female mice [14, chapter 2]. We found that the brain site with the highest level of colocalization of ERα and AR was the posterior amygdala.

The amygdala is a complex structure comprised of several subdivisions including the basolateral, central, cortical, medial, lateral, and the posterior amygdala. Each subdivision has a role in

different aspects of behavior and/or cognition [15-20]. The medial amygdala and the posterior amygdala have both been implicated in motivated behaviors associated with reproductive success, but whereas the medial amygdala's role is well defined, the posterior amygdala remains vastly understudied [18, 19, 21-28].

The posterior amygdala has been identified in several mammals including rabbits, shrews, mice, and rats [23,29-34]. Earlier studies defined this region as the cortico-amygdaloid transition area, first described in the shrew [30]. It was also referenced and described in rats as the posterior part of the medial nucleus of the amygdala, and later as the amygdalohippocampal area [23-25, 29].

In rats, the posterior amygdala densely projects to the lateral septum, medial preoptic area, and ventromedial nucleus of the hypothalamus, all relevant for reproductive function and behaviors [30]. In mice, activity of the projections from the posterior amygdala to the ventromedial hypothalamus was associated with male aggression [33-34]. Together, these findings suggest that in rodents the posterior amygdala projects to reproductive control sites and have a function in behaviors associated with reproductive success. However, whether PROKR2 expressing neurons participate in this circuitry has not been demonstrated.

In this chapter, we used molecular tracing techniques to define the projections and innervation targets of Prokr2 neurons of the posterior amygdala in adult male and female mice.

3.2 Methods

3.2.1 Animals

Adult (2-5 months old) male and female B6;SJL-*Prokr2*$^{em1(cre)Cfe}$ (ProkR2-Cre) eGFP-L10a mice were used for all experiments (JAX® stock #043846-JAX, JAX® stock # 024750, [35]. Cre-dependent eGFP-L10a mice were used to facilitate visualization of ProkR2-Cre cells and validate the injection sites and specificity of the viral vector. This mouse model was kindly provided by Dr. David Olson at the University of Michigan [36]. All mice were heterozygous for the *Prokr2*-Cre allele with one or two copies of the eGFP-L10a allele. Mice were maintained in a light- (12L:12D) and temperature- (21-23°C) controlled environment at the University of Michigan Animal Facility (ULAM) with free access to water and food. Mice were fed a phytoestrogen-reduced Envigo diet 2016 (16% protein/4% fat), except during breeding when they were fed a high protein phytoestrogen-reduced Envigo diet 2019 (19% protein/8% fat). A phytoestrogen-reduced diet is used in our laboratory to minimize the effects of exogenous estrogen in reproductive physiology [63-66]. All procedures and experiments were done in accordance with the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Michigan (protocol # PRO0000420). All mice were genotyped by extracting DNA from ear punch biopsies (RED Extract-N-Amp Tissue PCR Kit catalog no. XNAT, Sigma, St. Louis, MO, USA) and performing standard PCR (primers described in Table 1).

Table 3.1: Primers used for genotyping experimental animals.

Gene	Primer Sequence	Size: (bp)
ProkR2-Cre	F 5’ TCCCCACGGTAGTTGTGAAG 3’ R 5’ ATTGGTTGGTGTGGTTTGCAG 3’	Cre:353 Wt:499

	mt 5’ CAGCAGGTTGGAGACTTTCCT 3’	
eGFP-L10a (R26-loxSTOPloxeGFP-L10a)	F: 5’ GAG GGG AGT GTT GCA ATA CC 3’ F: 5’ TCT ACA AAT GTG GTA GAT CCA GGC 3’ R: 5’ CAG ATG ACT ACC TAT CCT CCC 3’	Wt: 300 Mt: 200

3.2.2 Stereotaxic Injections

A Cre-dependent ChannelRhodopsin-mCherry AAV (AAV-ChR2-mCherry) was used for mapping of neuronal projections (UNC Vector Core AAV-hSyn-hChR2(H134R)-mCherry, AAV5). Mice were anesthetized with 1.5–2% isoflurane (Fluriso; Vet One). The posterior amygdala injections in male mice were targeted 5 mm posterior to the rhinal vein, 2.1 mm lateral to midline, and 5.3 mm ventral to the dura-mater. In females, posterior amygdala injections were targeted 5 mm posterior to the rhinal vein, 2.1 mm lateral to the midline, and 5.1 mm ventral to the dura-mater. A volume of 100 nL of AAV-ChR2-mCherry was injected unilaterally using a pneumatic picopump (World Precision Instruments) as routinely performed in our lab [37-38]. The glass pipette was left in place for 3 min after injection to allow viral diffusion and avoid reflux. Carprofen (Rimadyl, 5 mg/kg, sc) was given preemptively and every 24h after surgery for two days as an analgesic. Mice were monitored for 10 days for recovery. After 3 weeks, mice were perfused, and brains were collected for histology.

3.2.3 Perfusions and Brain Histology

Mice were anesthetized with isoflurane and transcardially perfused with 0.9% diethylpyrocarbonate (DEPC)-treated 0.1M PBS followed by 10% neutral buffered formalin. Following perfusion, brains were collected and postfixed in 20% sucrose in 10% formalin for 2

h. Brains were cryprotected in 20% sucrose-DEPC-PBS overnight and sectioned in freezing microtome (Leica). Four series of 30 µm thick coronal sections were collected and stored at -20°C in RNAse-free cryoprotectant.

3.2.4 Immunohistochemistry

Sections were washed with 0.1M PBS to remove cryoprotectant and incubated in 0.3% hydrogen peroxide (H_2O_2) in Triton-PBS (0.25%) for 30 min. Sections were then incubated in anti-mCherry antibody made in rat (1:10,000, Thermo Fisher Scientific Cat# M11217, RRID: AB_2536611). The antibody solution was prepared with 3% normal donkey serum in 0.25% Triton X100 in 0.1M PBS and sections were incubated overnight at room temperature. The following day, sections were washed in 0.1M PBS and incubated in biotin-conjugated secondary donkey anti-rat antibody for 1 h (1:1,000, Jackson ImmunoResearch Laboratories), washed again in 0.1M PBS and incubated in Avidin-Biotin Complex (ABC) in 0.1M PBS (1:500, Vector Labs) for 1 h. The peroxidase reaction was performed using 0.05% 3,3'-diaminobenzidine (DAB) as chromogen with 0.01% hydrogen peroxidase. Following 0.1M PBS washes, sections were mounted on gelatin coated slides, dehydrated, delipidated in xylenes for 5 days and subjected to a silver-gold amplification procedure. Slides were counterstained with thionin, dehydrated, delipidated in xylenes and cover slipped with DPX.

3.2.5 Imaging

Injections sites were visualized in brightfield and projections in darkfield. Images were acquired using the upright microscope Zeiss Axio Imager M2 and a digital camera (Axiocam, Zeiss) and the Zen software. Images were organized in figures using Adobe Photoshop (Adobe Creative).

3.3 Results

3.3.1 Posterior Amygdala ProkR2-Cre neurons project to reproductive control sites

We performed stereotaxic injections of Cre inducible AAV-ChR2-mCherry into the posterior amygdala of ProkR2-Cre L10-GFP male and female mice to assess projections of posterior amygdala ProkR2 cells (Fig. 3.1). We obtained 3 male and 2 female mice with injections centered in the posterior amygdala, and 2 male and 3 female mice with injections in the posterior amygdala with spread of viral transduction to adjancent sites (Fig. 3.2 & Fig. 3.3). All samples were used for analyzing posterior amygdala ProkR2-Cre projections.

In males, dense projections were found in the lateral septum, ventral subdivision (Fig. 3.2, A), bed nucleus of the stria terminalis (Fig. 3.2, B), the medial preoptic area (Fig. 3.2, C), the ventrolateral subdivision of the ventromedial nucleus of the hypothalamus (Fig. 3.2, D) and the ventral premammillary nucleus (Fig. 3.2, E). Projections were also observed in the posterodorsal subdivision of the medial amygdala.

Females displayed similar projection pattern with dense innervation of the lateral septum (Fig. 3.3, A), bed nucleus of the stria terminalis (Fig. 3.3, B), the medial preoptic area (Fig. 3.3, C), the ventrolateral division of the ventromedial nucleus of the hypothalamus (Fig. 3.3, D) and the ventral premammillary nucleus (Fig. 3.3, E). We observed that the projections to all sites were noticeably different between sexes. Males exhibited denser projection patters compared to females. Minor contralateral projections were present in all sites in both sexes.

Analysis of the fibers morphology showed varicosities and terminal-like boutons, suggesting targeted innervation and synaptic contacts. Representative images for two reproductive control sites are shown (Fig. 3.4, A-B).

3.4 Discussion

In the present study we described the projection pattern of ProkR2-Cre neurons of the posterior amygdala in both male and female mice. Previous validation of this mouse model demonstrates that the posterior amygdala in both sexes express *Prokr2* in adulthood consistent with the reporter gene expression [35]. In fact, sexually dimorphic expression of ProkR2-Cre eGFP was observed in the posterior amygdala with males displaying a higher number of cells compared to females [35]. Aside from differences in successful AAV injection and viral expression, the sex difference in ProkR2 expression may explain the difference in density of projections between sexes.

Despite differing in ProkR2-Cre cell number in the posterior amygdala, both sexes display the same projection pattern to targeted sites. It is worth noting that the current approach cannot confirm whether the fibers at each reproductive control site form synapses or if they are fibers of passage. However, we do find dense varicosities and apparent terminal-like boutons in all these sites suggesting that posterior amygdala ProkR2 neurons specifically and densely innervate these sites.

The co-expression of gonadal steroid receptors along with the findings in this chapter favor a reproductive role for PROKR2 cells of the posterior amygdala [14, chapter 2]. ProkR2-Cre neurons are capable of sensing gonadal steroids milieu and likely process this information to the various brain regions implicated in a wide range of social and reproductive behaviors [14, chapter 2]. The lateral septum is a gonadal steroid dense region that regulates male sexual

behavior and aggression [40,41]. In females, research in mice have identify neurons in this area that are involved in regulating maternal behavior [42,43]. The lateral septum is also activated during sexual behavior [23-25] and lesions of the lateral septum result in impairment of male sexual behavior [44]. Lesions of the lateral septum in females impaired maternal behavior [42]. Projections to the bed nucleus of the stria terminalis are also similarly implicated in these previously mentioned behaviors [45-47].

The posterior amygdala ProkR2 neurons of both sexes also project to the medial preotic area, which is a crucial site for the regulation of the hypothalamo-pituitary-gonadal axis (HPG) axis. Among the distinct subdivisions of the preoptic area, is the anteroventral periventricular nucleus (AVPV) where lies the kisspeptin neurons [48]. Upstream of GnRH neurons, when stimulated, AVPV kisspeptin neurons activate the HPG axis and induce release of luteinizing hormone, necessary for ovulation in females [48-49]. Whether posterior amygdala ProkR2-Cre neurons directly innervate kisspeptin neurons needs evaluation. Together, the lateral septum, bed nucleus of the stria terminalis and the preoptic area, are part of a complex circuit activated by pheromone or olfactory cues and social/sexual behaviors [50-53].

The projections to the ventromedial hypothalamus specifically the ventrolateral subdivision reinforce a possible reproductive role for the ProkR2 neurons in the posterior amygdala. This site is associated with lordosis behavior in females and aggression in males; it also densely express gonadal steroids receptor [34, 50, 54-55]. Similarly, the premammillary nucleus is a critical site in the integration of internal (i.e., steroids milieu) and external environmental cues, including metabolic and relevant olfactory signals. It is also part of the hypothalamo-amygdala circuit that,

together with the medial amygdala, integrates olfactory cues in sex recognition [56-60]. *In situ* hybridization data shows Vglut1 mRNA in the posterior amygdala suggesting that neurons of the posterior amygdala use mainly glutamate neurotransmission in the terminal sites [61]. The strong projections of the ProkR2 neurons from the posterior amygdala to these sites suggest they may also be part of neural pathways linking the olfactory system and hypothalamic pathways for reproductive behaviors.

Previously, posterior amygdala projections were characterized in rats using *Phaseolus vulgaris* (PHAL), a standard anterograde tracer [30]. This study described projections to the same reproductive control sites we observed in our studies. PHAL, however, targets random neuronal populations. Though rats and mice share many similarities, it is important to keep in mind they also display many differences in neurochemistry and behavioral control [62]. Our molecular mapping and genetic approach identified the specific projections of posterior amygdala ProkR2 neurons. Together with chapter 2 [14], the findings described in this chapter strongly support the idea that these neurons have a role in the modulation of the HPG axis and motivated behaviors associated with reproductive function. To test this, we conducted functional studies described in chapter 4.

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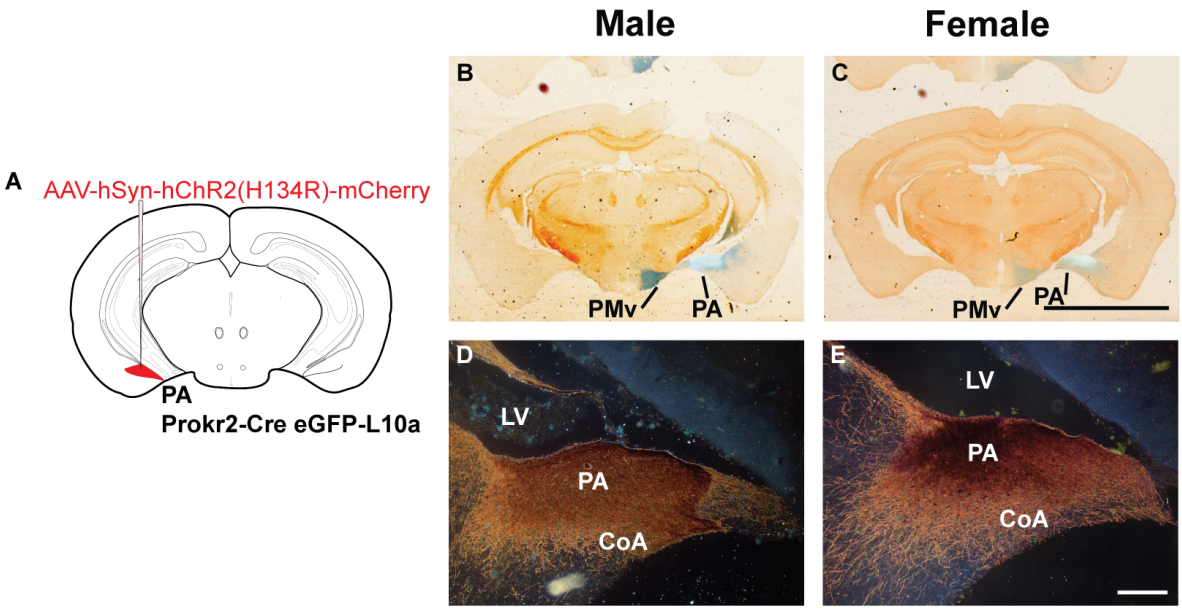

Figure 3.1: Successful stereotaxic injections in male and female brains A) Schematic illustration for the injection of cre dependent channelrhodopsin (Chr2)- used for mapping. (B) Example darkfield micrographs, of gold enhanced immunohistochemistry whole brain sections hits in the posterior amygdala showing visible Chr2-mCherry expression and Chr2–mCherry fibers from PA to the PMv nuclei in in males and (C) in females. (D) Darkfield images showing injection site in male and (E) female PA. *Brain atlas images adapted from Paxinos and Franklin, 2001* [67]. Abbreviations: CoA: cortical amygdala; LV: lateral ventricle; PA: posterior amygdala; PMv: ventral premammillary nucleus. Scale bar: B,C=500 µm, D,E=200 µm.

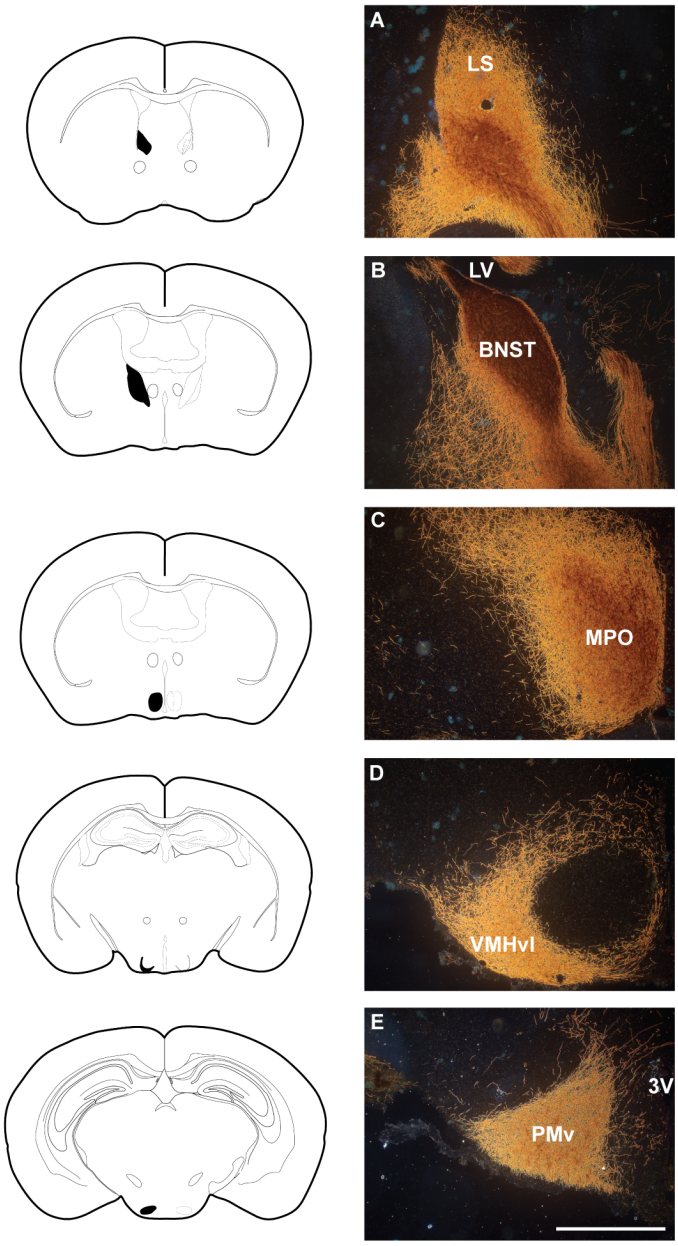

Figure 3.2: ProkR2-Cre projections from the posterior amygdala of males. Darkfield micrographs showing projections to the (A) lateral septum, (B) bed nucleus of the stria terminalis, to the (C) medial preoptic area, (D) ventromedial hypothalamus ventrolateral subdivision and (E) premammillary nucleus. Brain atlas reference image showing nuclei in whole brain section is represented on the left side. *Brain atlas images adapted from Paxinos and Franklin, 2001* [67]. Abbreviations: BNST: bed nucleus of the stria terminalis; LS: lateral septum; LV: lateral ventricle; MPO: medial preoptic nucleus; PA: posterior amygdala; PMv: ventral premammillary nucleus; VMHvl: ventromedial hypothalamus, ventrolateral subdivision; 3V: third ventricle. Scale bar: 500 μm.

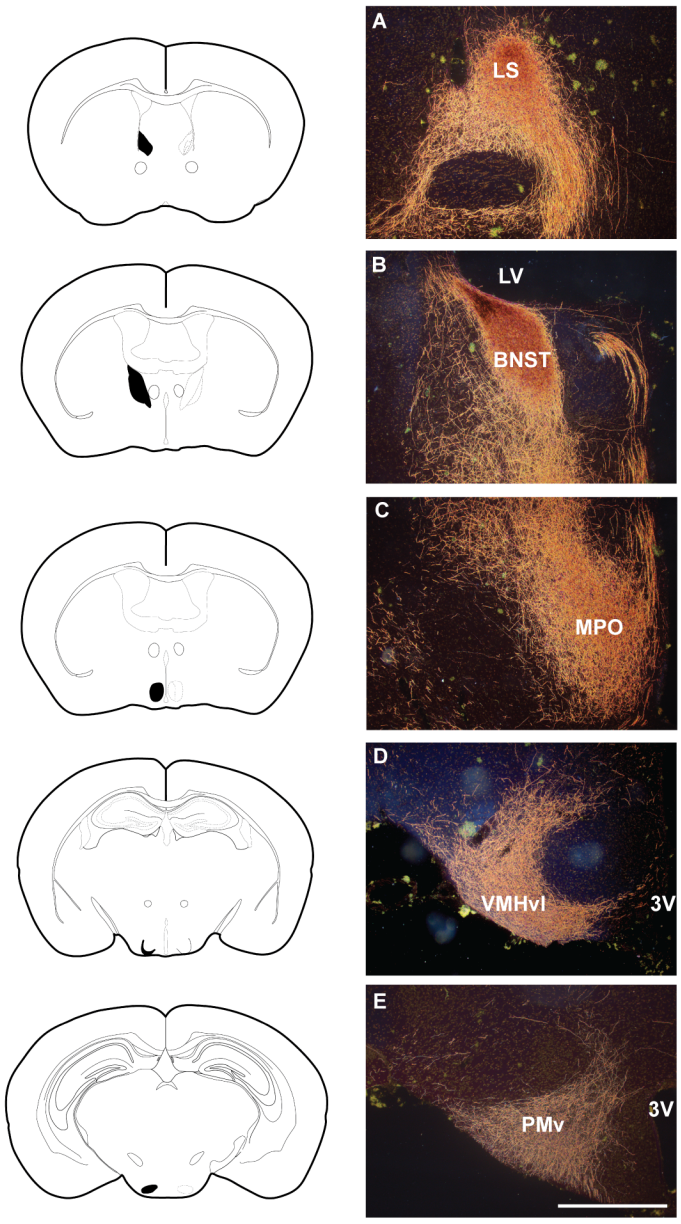

Figure 3.3: ProkR2-Cre projections from the posterior amygdala of females. Darkfield micrographs showing projections to the (A) lateral septum, (B) bed nucleus of the stria terminalis, to the (C) medial preoptic area, (D) ventromedial hypothalamus ventrolateral subdivision and (E) premammillary nucleus. Brain atlas reference image showing nuclei in whole brain section is represented on the left side. *Brain atlas images adapted from Paxinos and Franklin, 2001* [67]. Abbreviations: BNST: bed nucleus of the stria terminalis; LS: lateral septum; LV: lateral ventricle; MPO: medial preoptic nucleus; PA: posterior amygdala; PMv: ventral premammillary nucleus; VMHvl: ventromedial hypothalamus, ventrolateral subdivision; 3V: third ventricle. Scale bar: 500 µm

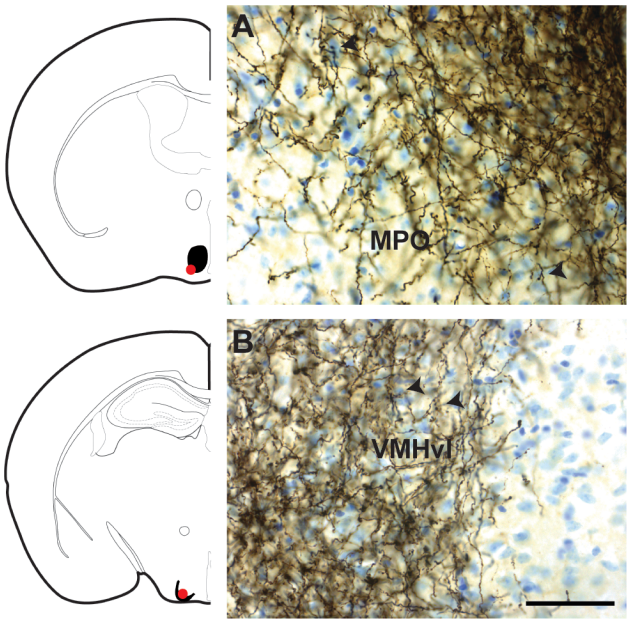

Figure 3.4: Representative images of dense varicosities observed in posterior amygdala ProrkR2-Cre projections to reproductive control sites. (A) Dense projections in the medial preoptic area show terminal-like boutons (B) Dense projections in the ventromedial hypothalamus, ventrolateral subdivision show terminal-like boutons. Brain atlas reference images on the left highlight magnified area in red. Arrows point to terminal-like boutons. *Brain atlas images adapted from Paxinos and Franklin, 2001* [67]. Abbreviations: MPO: medial preoptic area; VMHvl: ventromedial hypothalamus, ventrolateral subdivision. Scale bar: 50 μm

Chapter 4 Prokineticin Receptor 2 Neurons in the Posterior Amygdala Respond to Olfactory Cues and Stimulate Luteinizing Hormone Release

4.1 Introduction

Gonadotropin-releasing hormone (GnRH) deficiency and anosmia, caused by defective GnRH neuronal migration and olfactory bulb development, are characteristics of Kallmann Syndrome (KS) resulting in infertility [1-5]. Mutations in the prokineticin receptor 2 gene, *PROKR2*, have been identified in patients with KS [6-13]. Similarly, mice with global deletion of *Prokr2* have impaired GnRH neuronal migration and as a result are also infertile [14-16]. The precise mechanisms by which PROKR2 expressing neurons drive GnRH neuron migration have not been fully characterized. Aside from this developmental role, it is known that PROKR2 is highly expressed in the adult brain. Specifically, its dense expression in reproductive control sites warrants investigating PROKR2 expressing neurons in reproductive physiology.

The physiological role of PROKR2 neurons is not well understood in part due to lack of adequate animal models. Our lab overcame this by developing a ProkR2-Cre mouse model in which Cre recombinase expression is driven by the *Prokr2* promoter. With this mouse model, we mapped the distribution of PROKR2-expressing cells in the brain of both sexes. Using a GFP reporter line, we found sexually dimorphic expression of PROKR2 in many areas of the brain associated with reproductive control [17]. We previously showed that the posterior amygdala had ProkR2-Cre cells that coexpress gonadal steroids receptors relevant for successful reproduction,

i.e., ERα and AR in a sex-specific manner [18, chapter 2]. Additionally, using molecular mapping and viral vector injections we further mapped the posterior amygdala projections of ProkR2-Cre-expressing and found a dense innervation of forebrain sites associated with reproductive neuroendocrine function and motivated behavior in male and female mice [Chapter 3]. Altogether, our findings suggest that the PROKR2 containing neurons in the posterior amygdala have a role in reproductive neuroendocrine function.

In this study, we set out to determine if ProkR2-Cre cells are part of a neural network involved in reproductive behaviors and if they can act on the HPG axis for LH release in male mice. While we found similar projection patterns in male and female mice, we focused our studies on male mice as a first step in understanding the behavioral role of these neurons. We assessed if sexual behavior and/or conspecific odors induce Fos expression (a marker of cellular response) in ProkR2-Cre cells of the posterior amygdala. We further used chemogenetic approach, the designer receptor exclusively activated by designer drugs (DREADDs) coupled with Cre/loxP technology to determine if activation of ProkR2-Cre cells change circulating LH levels.

4.2 Methods

4.2.1 Animals

Adult (2-5 months old) male and female B6;SJL-*Prokr2*$^{em1(cre)Cfe}$ (ProkR2-Cre) eGFP-L10a mice were used for all experiments (JAX® stock #043846-JAX, JAX® stock # 024750, [17]). Cre-dependent eGFP-L10a mice were used to facilitate visualization of ProkR2-Cre cells and validate the injection sites and specificity of the viral vector. This mouse model was kindly provided by

Dr. David Olson at the University of Michigan [19]. All mice were heterozygous for the *Prokr2*-Cre allele with one or two copies of the eGFP-L10a allele. Mice were maintained in a light- (12L:12D) and temperature- (21-23°C) controlled environment at the University of Michigan Animal Facility (ULAM) with free access to water and food. Mice were fed a phytoestrogen-reduced Envigo diet 2016 (16% protein/4% fat), except during breeding when they were fed a high protein phytoestrogen-reduced Envigo diet 2019 (19% protein/8% fat). A phytoestrogen-reduced diet is used in our laboratory to minimize the effects of exogenous estrogen in reproductive physiology [60-63]. All procedures and experiments were done in accordance with the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Michigan (protocol # PRO0000420). All mice were genotyped as described in chapter 3.

4.2.2 Mating Behavior

Sexually experienced males and females of proven fertility were used as experimental mice. Males were individually housed in cages with wired mesh tops for at least two days. A recording set up in a closed room using ZOSI 1080P CCRV Cameras was used to record mating behavior such as mounts, intromission and ejaculation as described in McGill 1962 and André et al., 2021 [20-21]. We performed vaginal lavages on females for two weeks to assess estrous cycle stage, select normal cycling females and to determine the proestrus day, when females are expected to be receptive [22-24]. A proestrus female was moved to the male cage one hour after lights off, behavior was recorded, and ejaculation was determined by the increased thrusting and spasmodic rear leg movements as described in (McGill, 1962) and (André et al., 2021) [20-21]. 90 min after ejaculation, male brains were collected for histology.

4.2.3 Soiled Bedding Odor Test

Soiled bedding for all experiments came from either normally cycling females that were individually housed or from individually housed males for one week prior to experiments. Cotton nestlets were added to cage bedding. Experimental animals were either sexually naïve or sexually experienced and were also individually housed. Experimental and control animals received daily handling for 1 h before lights off for 6 days to reduce stress on the day of experiment. On day 7, mice were divided into two groups: those exposed to clean bedding or those exposed to soiled bedding. For males exposed to female soiled bedding, 1 h before lights off, males were moved to either a clean or a soiled bedding cage. For males exposed to male soiled bedding, the cotton bedding was added to home cage of males instead of transferring males to a soiled cage to reduce Fos expression related to territoriality and aggression and focus on odor stimulation [25-26]. Following 90 min, all animals were perfused, and brains were collected as previously described.

4.2.4 Immunofluorescence

Sections were washed using 0.1M PBS to remove cryoprotectant and were incubated in primary anti-GFP (1:5,000, AvesLabs, catalog #GFP-1010; RRID: AB_2307313) and/or anti-Fos (1: 2,000, Millipore Cat# ABE457, RRID: AB_2631318) antibodies in 0.25% Triton in 0.1 M PBS and 3% donkey serum, overnight at room temperature. The next day sections were washed using 0.1M PBS and incubated in donkey anti-rabbit Alexa Fluor 594 (Thermo Fisher Scientific Cat# A-21207, RRID: AB_141637) for 1 h. Sections were then washed using 0.1 M PBS and incubated in donkey anti-chicken Alexa Fluor 488 (Thermo Fisher Scientific Cat# A-11029, RRID: AB_2534088) for 1 h. Following a last wash, sections were mounted on gelatin coated

slides and coverslipped with Fluoromount™ (Southern Biotechnology Associates, Birmingham, AL, USA).

4.2.5 Stereotaxic Injection

Adult mice were used for all surgeries. A Cre-dependent AAV hM3Dq-mCherry was used for remote activation of the posterior amygdala ProkR2-Cre neurons. pAAV-hSyn-DIO-hM3D(Gq)-mCherry was a gift from Bryan Roth (Addgene plasmid # 44361 ; http://n2t.net/addgene:44361 ; RRID:Addgene_44361 [27]. Mice were anesthetized with 1.5–2% isoflurane (Fluriso; Vet One). Posterior amygdala injections in male mice were targeted 5 mm posterior to the rostral rhinal vein, ±2.1 dmm lateral to midline, and 5.3 mm ventral to the dura-mater. In females, posterior amygdala injections were targeted 5 mm posterior to the rostral rhinal vein, ±2.1 mm lateral to the midline, and 5.1 mm ventral to the dura-mater. A volume of 100 nL of AAV was injected bilaterally using a pneumatic picopump (World Precision Instruments) as routinely performed in our lab [28-29]. The glass pipette was left in place for 3 min after injection to allow viral diffusion and avoid reflux. Carprofen (Rimadyl, 5 mg/kg, sc) was given preemptively and every 24h after surgery for two days as an analgesic. Mice were monitored for 10 days for recovery.

4.2.6 Posterior Amygdala ProkR2-Cre Cell Activation and Culex Automated Blood Collection

Three weeks after stereotaxic injections, adult male mice were transferred to the Michigan Mouse Metabolic Phenotyping Center (MMPC, NIDDK) and underwent jugular vein and carotid artery catheterization. Following 4 days of recovery, mice were connected to a Culex automated blood collection machine [30]. Blood samples were collected at 15-min intervals for 30 min prior to clozapine N-oxide (CNO) intraperitoneal injection (0.5 mg/kg), and 1 h following CNO

injection. Mice remained in the chambers for an additional hour after which brains were collected for histology (2hrs post CNO).

4.2.7 Perfusion and Brain Histology

Mice were anesthetized with isoflurane and transcardially perfused with 0.1M PBS followed by 10% neutral buffered formalin. Following perfusion, brains were collected and postfixed in 20% sucrose in 10% formalin for 2 h. Brains were cryoprotected in 20% sucrose-DEPC-PBS overnight and sectioned in freezing microtome (Leica). Four series of 30 μm thick coronal sections were collected and stored at -20 °C in RNAse-free cryoprotectant.

4.2.8 Luteinizing Hormone Analysis

Blood samples were processed, and sera were shipped to University of Virginia's Ligand Assay and Analysis Core. Samples were analyzed by the Core using an ultrasensitive LH ELISA assay. This ELISA assay method has an average reportable range of 0.016 to 4 ng/mL [31].

4.2.9 Dual-label Immunoperoxidase:

Sections were washed with 0.1M PBS to remove cryoprotectant and were incubated in 0.3% hydrogen peroxide (H_2O_2) in Triton-PBS (0.25%) for 30 min. Sections were then incubated in anti-Fos (1: 5,000, Millipore Cat# ABE457, RRID:AB_2631318) in 0.25% Triton in 0.1 M PBS and 3% donkey serum, overnight at room temperature. The following day, sections were washed in 0.1M PBS and incubated in biotin-conjugated secondary donkey anti-rabbit antibody for 1 h (1:1,000, Jackson ImmunoResearch Laboratories), washed again in 0.1M PBS and incubated in Avidin-Biotin Complex (ABC) in 0.1M PBS (1:500, Vector Labs) for 1 h. The peroxidase reaction was performed using 0.05 % 3,3'-diaminobenzidine (DAB) as chromogen with 0.01 % hydrogen peroxidase and 0.05 % nickel ammonium sulfate as chromogens. Following 0.1 M

PBS washes and another 30 min 0.3% hydrogen peroxide (H_2O_2) incubation, sections were incubated in chicken anti-GFP primary antibody (1:20,000, AvesLabs, catalog #GFP-1010; RRID:AB_2307313) anti-mCherry antibody made in rat (1:5,000, Thermo Fisher Scientific Cat# M11217, RRID:AB_2536611) to label hM3Dq positive cells. Donkey anti-rat Alexa Fluor 594 (Thermo Fisher Scientific Cat# A-21209, RRID: AB_2535795) and donkey anti-chicken Alexa Fluor 488 (Thermo Fisher Scientific Cat# A-11029, RRID:AB_2534088) were used as secondaries following the immunofluorescence protocol previously described. Following the last 0.1M PBS washes, sections were mounted on gelatin coated slides, coverslipped with Fluoromount^{TM} (Southern Biotechnology Associates, Birmingham, AL, USA).

4.2.10 Statistics

Data are reported as the mean ± SEM. Prism, version 9 (GraphPad Software Inc.) was used for analysis. A Shapiro–Wilk test (significance alpha 0.05) was used to test normal distribution of data. An unpaired *t* test with Welch's correction was used for normally distributed data and a Mann–Whitney non-parametric test was used for non-normally distributed data.

4.2.11 Image Capture

All images were captured and processed as described in chapter 2.

4.3 Results

4.3.1 ProkR2-Cre neurons of the posterior amygdala respond to conspecific opposite sex olfactory cues.

Mating behavior

We exposed sexually-experienced males to either a clean cage (control condition) or a proestrus female that was normally cycling to determine Fos immunoreactivity (Fos-ir) in the posterior amygdala ProkR2-Cre cells in response to sexually relevant stimuli. Fos expression of males exposed to a clean cage (Fig. 4.1, A-B) or a proestrus female (Fig. 4.1, C-D) was assessed (n=3 for each group). After mating with a female (observed intromission and ejaculation), we observed higher Fos-ir in the posterior amygdala of males compared to those exposed to an empty cage (23.33 ± 3.75 Fos-ir cells *vs* 5 ± 2.52 Fos-ir cells, $p = 0.02$. (Fig. 4.1, E). The percentage of Fos-positive neurons that coexpress ProkR2-Cre eGFP-L10a was not different as a subset of Fos-ir cells observed in the control group coepressed GFP (Fig. 4.1, F).

Males exposed to female odor

Because sexual behavior has many components that could induce Fos-ir in male mice, we assessed if female odors only could be responsible for the effects observed. We performed female soiled bedding exposure tests on sexually experienced males to determine Fos-ir in the posterior amygdala ProkR2-Cre cells. Males were exposed to clean bedding (Fig. 4.2, A-B) and soiled bedding from cycling females (Fig. 4.2, C-D, n=6 per group). Exposure to female soiled bedding significantly increased Fos in ProkR2-Cre neurons of the posterior amygdala (26.67± 4.014 Fos-ir cells vs 6.33 ± 1.606 Fos-ir cells, p=0.002, Fig. 4.3, E). An estimated 45.9% of Fos-ir cells coexpress ProkR2-Cre eGFP-L10a and were significantly higher compared to controls (p=0.002, Fig. 4.3, F).

Male exposed to male odor

We performed male soiled bedding exposure tests with sexually experienced males to determine Fos activation of posterior amygdala ProkR2-Cre cells. Males were exposed to clean bedding (Fig. 4.4, A-B) or soiled bedding from individually housed males (Fig. 4.4, C-D n=5 clean bedding, n=4 soiled bedding), and Fos immunoreactivity was assessed after exposure. Exposure to male soiled bedding did not increase Fos-ir in the posterior amygdala compared to clean bedding (8.75 ± 1.109 Fos-ir cells vs 3 ± 1.897 Fos-ir cells, p=0.1, Fig. 4.4, E). Of the few Fos-ir cells present, about half co-express ProkR2-Cre eGFP-L10. (Fig. 4.4, F).

4.3.2 Chemogenetic activation of ProkR2-Cre neurons of the posterior amygdala increase LH levels in male mice.

We performed bilateral stereotaxic injections of Cre inducible hM3Dq-mCherry into the posterior amygdala of ProkR2-Cre eGFP-L10a of males and collected blood samples (Fig. 4.5, A-B). Successful injections were determined by mCherry immunoreactivity (ir) in the posterior amygdala (Fig. 4.5, A-D). Eight of 11 mice with injections targeting the PA had an increase of LH after CNO administration (Fig. 4.6, A). Of those 5 had some spread of viral transduction to adjacent sites (Fig. 4.6, B). Injections outside of the posterior amygdala were considered misses and used as controls. Brains with missed injections had no detectable mCherry expression suggesting stereotaxic injections were either in brain sites without Cre expression or outside of the brain. The control mice with injections outside of the posterior amygdala showed no increase in LH after CNO (Fig. 4.6, C). To validate the injections, we also used Fos-ir. Statistical analysis revealed a different in LH between miss and hit injections to the posterior amygdala, $p<0.05$ (Fig. 4.6 D). We found a positive correlation between Fos-positive neurons in the posterior amygdala and LH levels, $p<0.05$. (Fig. 4.6, E). We also found Fos expression in the posteroventral subdivision of the medial amygdala (MePV) across all groups, including those

with contamination of adjacent nuclei (that includes MePV). However, no correlation between increase in LH values and MePV Fos-ir was observed.

4.4 Discussion

Social and reproductive behaviors in mammals such as rats, mice, and other rodents, rely on complex neural pathways connecting the olfactory system to the amygdala. The amygdala is divided into several subdivisions that process olfactory cues and drive motivated behaviors [32]. The amygdala is considered a critical chemosensory integration center for social behaviors and reproductive success [33-34]. The rodent olfactory system processes olfactory cues via the main and the accessory olfactory paths and convey odor information to the bed nucleus of the stria terminalis, the medial amygdala, and subdivisions of the cortical amygdala [35-40]. The subdivisions of the amygdala then project directly to the hypothalamus to modulate social behaviors and induce neuroendocrine responses [41-45].

Odor stimulation, lesions in brain nuclei and behavioral assays coupled with measuring reproductive hormone levels have been critical for mapping the olfactory system, amygdala, and hypothalamic function. Odorants in urine, soiled bedding and exposure to potential mates increases circulating LH and testosterone levels in mice [46-49]. Lesions of the ventral premammillary nucleus prevent a rise in LH following exposure to soiled bedding demonstrating that intact connections to the PMv are required for LH release [50]. Lesions of the medial amygdala disrupt lordosis behavior in female mice and sexual behavior in male rats [51-52]. Additionally, electrochemical stimulation of the cortical and medial amygdala facilitates LH release [53]. These studies by design target the entire population of neurons in specific nuclei and, in some approaches, they also lesion fibers of passage. The interpretation of the results,

therefore, may be incomplete as they do not address mechanisms, type of cells or chemical phenotypes of targeted populations. Regardless, they have been valuable in identifying the amygdala and hypothalamic pathways linking olfactory stimulation and reproductive behaviors.

Our results are in line with the olfactory-amygdala-hypothalamic function. The findings in chapter 3 showing the projections of ProkR2-Cre neurons of the posterior amygdala, place these cells in the neural circuits previously described. In this chapter, we show that ProkR2-Cre neurons in the posterior amygdala are responsive to opposite sex odors and appear to be primarily associated with sex recognition via olfactory input considering the percentage of Fos-ir cells expressing ProkR2-Cre GFP is similar comparing odor exposure and sexual behavior. Reinforcing this interpretation is the fact that we found a more robust response with female soiled bedding compared to male soiled bedding exposure indicating that ProkR2-Cre neurons are responsive to opposite sex odors and may play a role in social recognition of opposite sex mates. This is consistent with previous research implicating the posterior amygdala in mating and soiled bedding test in mice using Fos-ir as a metric [54-57].

Other studies in mice have looked at the posterior amygdala in male-male interaction but with social and resident-intruder assays allowing for physical interactions [58]. Additionally, they assessed a subset of posterior amygdala cells, the ERα positive cells. Those projecting to the medial preoptic nucleus were activated during mating and were necessary and sufficient for sexual behaviors in males, while those projecting to the ventromedial hypothalamus were activated during intermale aggression [59]. Our studies agree with these findings as we observed a significant increase in circulating LH levels following stimulation of the ProkR2-Cre neurons

of the posterior amygdala. We also found a positive correlation between peak LH values and number of Fos immunoreactive neurons in the posterior amygdala. The variability of LH values from mouse to mouse may be due to several aspects. The experimental approach we used requires injection of a Cre-dependent AAV bilaterally into a very small area. Although we have maintained consistent injection volume and stereotaxic coordinates, small differences are expected. Distinct subsets and number of Prokr2-Cre cells may be targeted in different mice, inducing a variable response.

We learned from our findings in chapter 3 that ProkR2-Cre cells from the posterior amygdala project to many brain sites. It is possible that Prokr2-Cre neurons are chemically heterogeneous and that subpopulations project to distinct brain sites. In fact, we showed in chapter 2 that only a subpopulation of ProkR2-Cre neurons in the posterior amygdala coexpresses gonadal steroids receptors [18]. Our experimental approach does not allow for dissociation between specific subsets of ProkR2 neurons, therefore further studies are necessary to define whether these neurons have distinct neural circuitry and function.

With regards to female reproductive physiology, more work needs to be done to identify the behavioral role, if any, and whether the projection patterns in females are also indicative of a neural circuit capable of affecting LH secretion.

4.5 References

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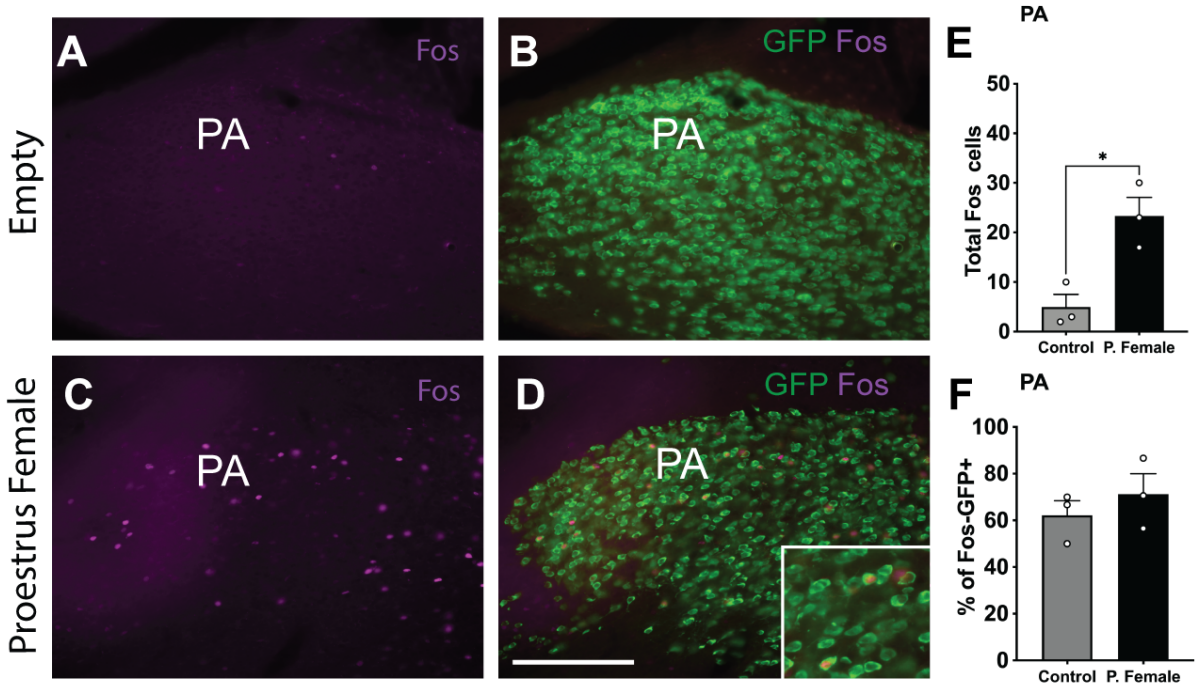

Figure 4.1: Fos immunoreactivity (ir) in the posterior amygdala following sexual behavior. (A-B) Fluorescent micrographs showing Fos-ir (A) and (B) merge with posterior amygdala ProkR2-Cre eGFP-L10a cells of male exposed to an empty cage. Fluorescent micrographs for (C) Fos-ir and (D) merge with posterior amygdala ProkR2-Cre eGFP-L10a of male exposed to a proestrus female. (E) Quantification of Fos following exposure to an empty cage or a proestrus female. (F) Percentage of Fos-ir cells coexpressing ProkR2-Cre eGFP following sexual behavior. Abbreviation: PA: posterior amygdala. Scale=200 μm.

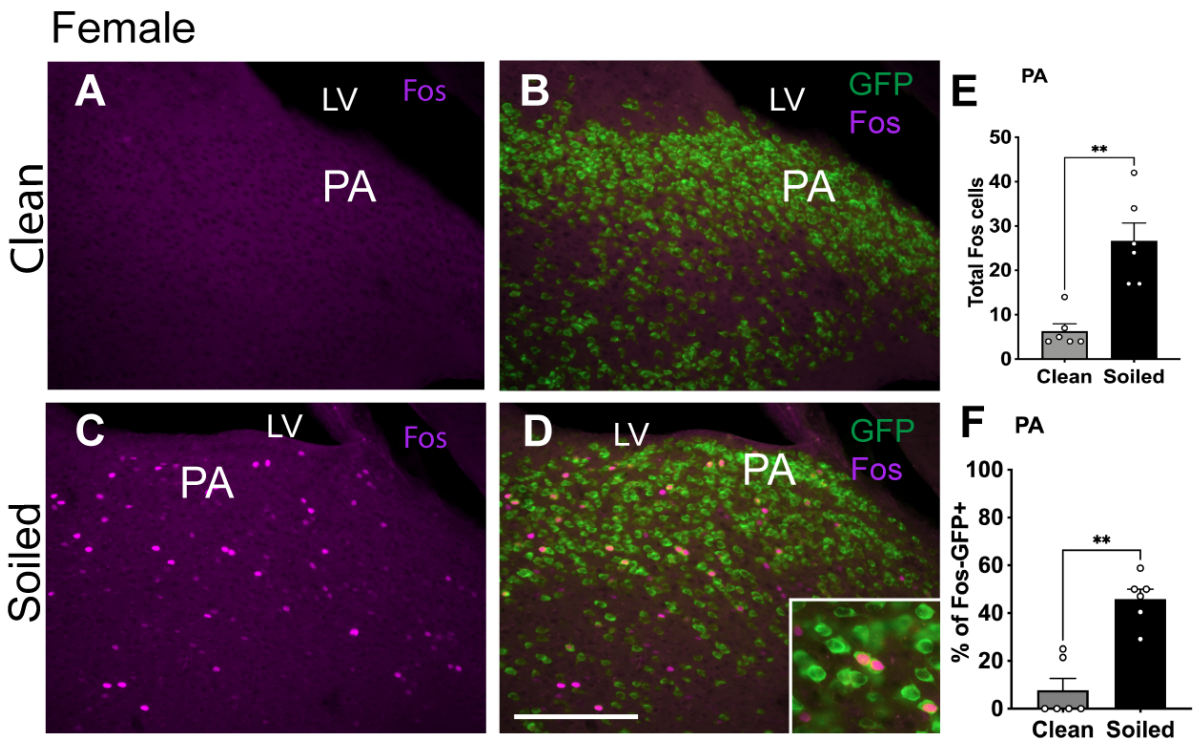

Figure 4.2: Fos immunoreactivity (ir) in the posterior amygdala of males following exposure to female soiled bedding. Fluorescent micrographs showing Fos-ir following (A) clean bedding and (B) merge with posterior amygdala ProkR2-Cre eGFP-L10a cells of male exposed to clean bedding. Fluorescent micrographs for (C) Fos-ir and (D) merge with PA ProkR2-Cre eGFP-L10a cells of male exposed to female soiled bedding. (E) Quantification of Fos and (F) percentage of Fos-ir cells with GFP following bedding exposure, p=<0.005. Abbreviations: LV: lateral ventricle; PA: posterior amygdala. Scale=200 µm.

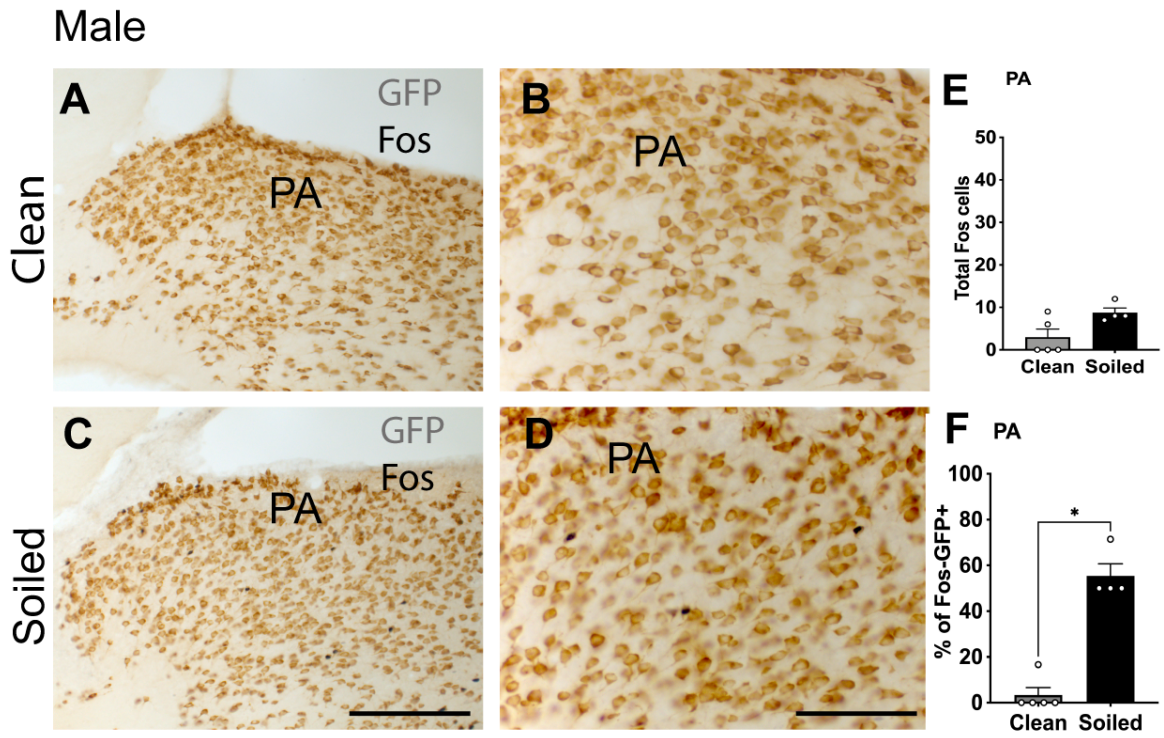

Figure 4.3: Fos immunoreactivity (ir) in the posterior amygdala of males following exposure to male soiled bedding. (A-B) DAB and Nickel staining for GFP and Fos in the posterior amygdala, respectively, following exposure to clean bedding (C-D). DAB and Nickel staining for GFP and Fos in the posterior amygdala, respectively, following exposure to male soiled bedding. Quantification of (E) Fos and (F) percentage of Fos-ir cells with GFP following bedding exposure. Abbreviation: PA: posterior amygdala. Scale bar: A,C=200 µm, B,D=100 µm

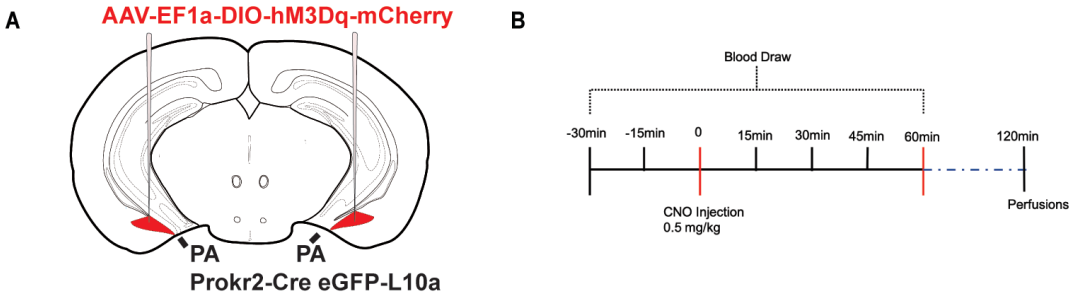

Figure 4

Figure 4.4: Schematic illustration of the experimental design. (A) Bilateral injection of Cre dependent hM3Dq into the posterior amygdala. (B) Timeline for blood sample collections using automated culex sampling.

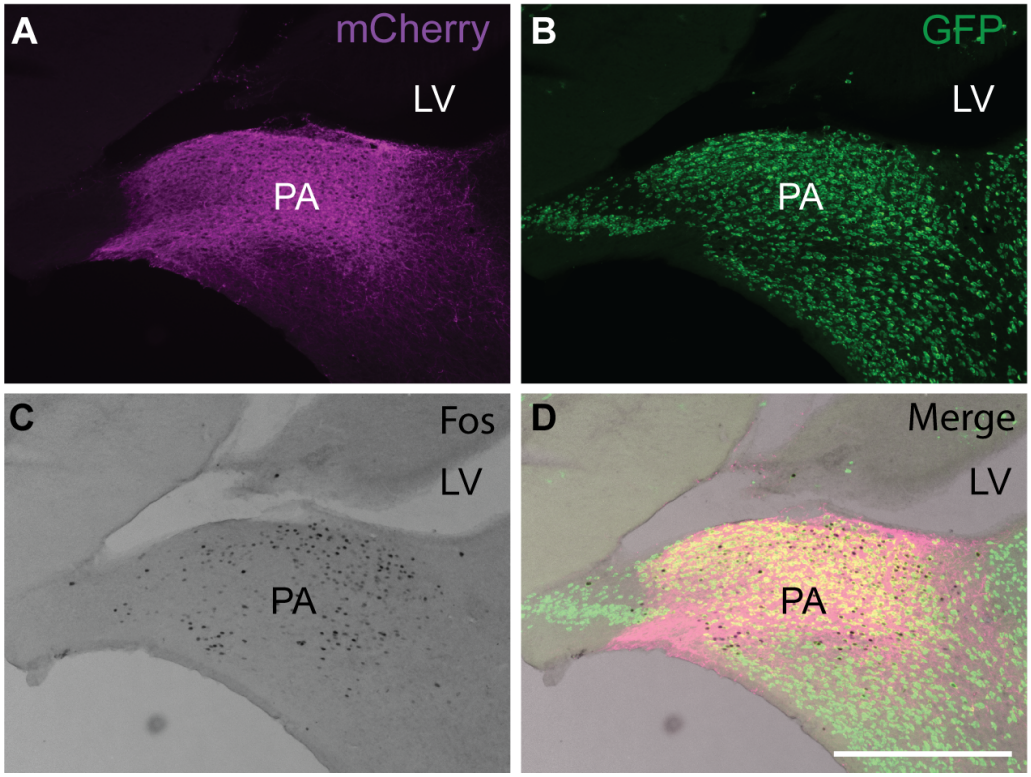

Figure 4.5: ProkR2-Cre neurons of the posterior amygdala (PA) express hM3Dq-mCherry. (A) Fluorescent micrographs showing successful expression of hM3Dq-mCherry in the posterior amygdala. (B) Fluorescent micrographs for GFP showing distribution of ProkR2-Cre eGFP-L10a cells. (C) Brightfield micrographs showing expression of Fos-ir following clozapine N oxide (CNO) injection. (D) All channels merged showing Fos-ir within hM3Dq expressing ProkR2-Cre eGFP-L10a cells. Abbreviations: LV, lateral ventricle. Scale=200 μm

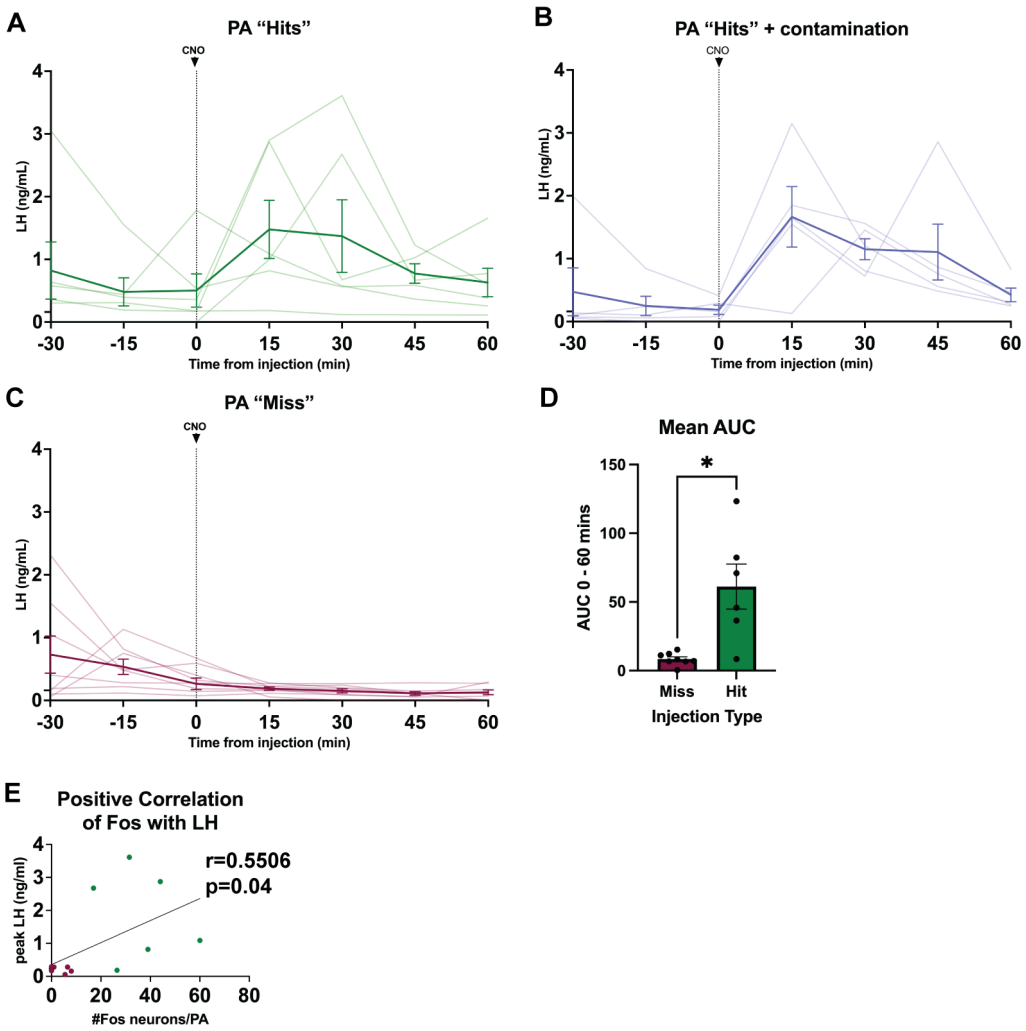

Figure 4.6: Chemogenetic activation of ProkR2-Cre neurons of the posterior amygdala (PA) induces luteinizing hormone (LH) secretion. (A) CNO increases LH in males with successful hM3Dq injection in the posterior amygdala. Each green line represents one mouse. The dark green line represents mean ± SEM of all data combined (n= 6 mice). (B) CNO increases LH in males with hM3Dq injections centered in the posterior amygdala with contamination of adjacent nuclei (posteroventral subdivision of the medial amygdala (MePV) and cortical amygdala). Dark purple line represents mean ± SEM of all data combined (n= 5 mice). (C) CNO administration does not increase LH secretion in males with injections outside of the posterior amygdala. Each light magenta lines represents one mouse. The dark magenta line represents mean ± SEM of all data combined (n = 8 mice). (D) Mean area under the curve is significantly higher in males with posterior amygdala hit. $p<0.05$. (E) Positive correlation between peak LH values and number of Fos-ir neurons in the PA. Magenta represents a mouse with a missed injection to the posterior amygdala. Green represents a mouse with a hit injection to the posterior amygdala.

Chapter 5 Conclusions

The PROK2/PROKR2 system is critical for development and migration of GnRH neurons. Genetic studies in humans showed that mutations in this system often result in reproductive deficits and animals models confirmed that PROK2/PROKR2 genes are necessary for sexual maturation. However, beyond involvement in GnRH neuronal migration, the role of PROKR2 expressing cells in adult reproduction had not been investigated due to animal models limitations.

The ProkR2-Cre mouse model allowed us to selectively label and target ProkR2 cells of the posterior amygdala. The expression of Cre-induced reporter gene is consistent with the *Prokr2* gene expression in adult mice. We can reliably use this mouse model to target specific *Prokr2*-expressing cell populations. Using this mouse model, we focused our studies on adult reproduction in a mouse with an intact HPG axis. In chapter 2, we showed that the posterior amygdala of both sexes is the only brain site with significant and consistent colocalization of ProkR2-Cre and gonadal steroid receptors, i.e., AR and ERα. These findings indicate that ProkR2-Cre cells of the posterior amygdala can sense androgens and estradiol and are likely involved in reproductive behaviors dependent on the gonadal steroid milieu. We then built on these findings in Chapter 3 and identified the projection sites. We found that the ProkR2-Cre cells of the posterior amygdala of both males and females project to sites associated with neuroendocrine responses and motivated behaviors. We performed a series of sexual behavior

and conspecific odor exposure (i.e., soiled bedding tests) and found that ProkR2-Cre cells are likely involved in the social recognition of opposite sex in males. We further hypothesized that this response was part of the circuitry that integrates sensory stimulation and neuroendocrine responses. To test this hypothesis, we use the DREADDs approach and found that activation of Prokr2-Cre cells of the posterior amygdala in males resulted in increase of circulating LH. We found a positive correlation between number of Fos-positive cells in the posterior amygdala and peak LH levels in males. These findings demonstrate that ProkR2-Cre cells of the posterior amygdala are part of a neural circuitry capable of responding to gonadal steroids, are activated by female olfactory cues (in males) and are apt to stimulate LH secretion in the presence of an opposite sex mate allowing for reproductive success.

5.1 Limitations and Future Studies

In chapter 2 we identified subpopulations of ProkR2-Cre cells that express AR or ERα. We know from studies in other mammalian species that the posterior amygdala has a subpopulation of neurons that express both receptors. Due to experimental constraint, we were not able to define if a subset of ProkR2-Cre in the posterior amygdala coexpress AR and ERα and whether they project to distinct forebrain sites.

The use of a Cre-dependent AAV-ChR2-mCherry allowed us to selectively infect ProkR2-Cre cells and label their projections. Our findings indicate similar projection patterns in male and female mice. However, our approach does not identify the specific neuronal targets and synaptic contacts. Use of high magnification tools (e.g., electron microscopy), optogenetic-guided neuronal circuit identification, and/or synaptic markers coupled with high resolution microscopy

are necessary to distinguish synaptic contacts from fibers of passage. Futures studies should also evaluate the brain sites that innervate *Prokr2*-expressing cells of the posterior amygdala, that is brain sites expressing the ligand prokineticin 2. These findings will further inform the understanding of the neural circuitry of the PROK2/PROKR2 system.

The soiled bedding exposure tests were done using sexually-experienced male mice given the robust literature showing an increased response to soiled bedding after sexual encounters. Since our objective was identifying the potential activation of ProkR2-Cre cells in response to olfactory cues, this approach was appropriate. Additional studies, however, must be done to fully characterize the role of Prokr2-Cre response to odor in sexually naïve mice. Additionally, further studies are needed to assess the role of Prokr2-Cre cells of the posterior amygdala in female mice. Investigating maternal behavior and other social behaviors is warranted.

We were able to selectively deliver and express 'excitatory' DREADDs in neurons of the posterior amygdala in males. This method allowed us to activate ProkR2-Cre cells and measure their impact on circulating LH. Though we found there was a positive correlation between peak LH values and number of Fos immunoreactive cells in the posterior amygdala, the increase in LH was variable from mouse to mouse, explained at least in part by difference in targeted sites, the specific subpopulation of neurons and number of cells that incorporated the AAV (discussed in Chapter 4). Planning experiments targeting activation of a subset of neurons (i.e., ProkR2-Cre cells projecting to the VMH vs those projecting to the PMv) would provide insights into specific neural pathways capable of stimulating LH secretion. These studies will aid in clarifying our current results.

Our studies activate ProkR2-Cre expressing neurons and, therefore, do not provide insights into the direct impact of the PROK2/PROKR2 signaling on LH secretion. Use of 'excitatory' DREADDs allows for stimulation of cells and for peptides and neurotransmitters to exert their effects on downstream targets. Investigating the impact of PROK2/PROKR2 signaling would require development of a mouse model harboring loxP sites flanking the *Prokr2* gene and designing experiments using AAV's selectively ablate *Prokr2* in those cells. A similar approach could be done using a second mouse model where the *Prok2* gene is floxed. These two approaches combined with neuroendocrine and behavioral tests would identify the impact of PROK2/PROKR2 signaling on reproductive behaviors.

Selective inhibition of ProkR2-Cre cells of the posterior amygdala is underway in our laboratory. We will assess if inhibition of these neurons will block the LH rise induced by female soil-bedding exposure. If we find this to be the case, we will have demonstrated that the ProkR2-Cre cells of the posterior amygdala are required for the rise in LH following opposite sex odor exposure. On the other hand, if we find the LH secretion is intact after ProkR2-Cre cells of the posterior amygdala are inhibited, we will conclude that ProkR2-Cre cells are sufficient but not required for male response to female odors. We will attribute this observation to the existence of redundancy in the neural pathways controlling the reproductive functions and species survival.