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The impact of postpubertal overexpression of Makorin ring finger protein 3 (MKRN3) on arcuate kisspeptin neurons in a novel mouse model

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BOSTON UNIVERSITY

ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Thesis

**THE IMPACT OF POSTPUBERTAL OVEREXPRESSION OF MAKORIN RING
FINGER PROTEIN 3 (MKRN3) ON ARCUATE KISSPEPTIN NEURONS IN A
NOVEL MOUSE MODEL**

by

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B.S., University of New England, 2022

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DEDICATION

I would like to dedicate this work to my parents – Jeanmarie and Alex. Without their unwavering support I would not be able to make it through the rigor of higher education.

I owe my past, present, and future successes to them as much as I do myself.

ACKNOWLEDGMENTS

I would like to thank Jacqueline Capuano, Joseph McElwee, Khushboo Patel, Olivia Lingren, and Sara Hanna for helping me get through countless hours spent in the library. I'd like to thank Dr. Stephanie Roberts for introducing me to the complex and captivating world of endocrinology. I'd also like to acknowledge the Kaiser and Navarro labs for allowing me the space to grow into new skill sets and new friendships.

**THE IMPACT OF POSTPUBERTAL OVEREXPRESSION OF MAKORIN RING
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SAMANTHA MARIA BLAU

ABSTRACT

Background: Makorin ring finger protein 3 (MKRN3) is thought to function as a ‘brake’ in the initiation of puberty, inhibiting gonadotropin-releasing hormone secretion. Loss-of-function mutations in *MKRN3* are the most common genetic cause of central precocious puberty. In female mice, *Mkfn3* deficiency has been demonstrated to advance the onset of puberty and *Mkfn3* overexpression results in delayed puberty. *Mkfn3* has been demonstrated to inhibit activators of GnRH secretion, including neurokinin B and kisspeptin in the arcuate nucleus.

Objective: This project aims to investigate the effects of *Mkfn3* overexpression in the arcuate nucleus of postpubertal female mice. This novel mouse model will be used specifically study the inhibitory effects of *Mkfn3* on kisspeptin neurons and explore the developmental windows in which *Mkfn3* acts on the hypothalamic-pituitary-gonadal axis.

Methods: A novel adeno-associated virus (AAV) to selectively overexpress *Mkfn3* in co-secreting kisspeptin, neurokinin B and dynorphin neurons will be introduced in the arcuate nucleus of postpubertal Kiss1-Cre female mice via bilateral stereotaxic injection. To generate the selective AAV-injected mouse model, this project first focused on generating the plasmid with the appropriate constructs for AAV packaging. To create

AAV-DIO-Mkrm3, pAAV-DIO-mCherry was modified to include *Mkrm3* and IRES, which were inserted via oligonucleotide synthesis. Plasmid prep kits were used to isolate and purify DNA. Plasmid constructs were validated using restriction enzyme digestion and Sanger sequencing. Prior to AAV packaging, DNA was isolated using EndoFree plasmid prep kits and validation was reperformed.

Results: From the standard plasmid prep kit derived DNA, the digestion of pAAV-DIO-mCherry, EcoRI and BamHI created linearized fragments at approximately 6.0 Kb, as expected. pAAV-DIO-Mkrm3, EcoRI created fragments at 1.4 Kb and 7.0 Kb and SmaI created visible fragments at approximately 2.2 Kb, 2.6 Kb, and 3.6 Kb, as anticipated. Subsequently, the digestion with the EndoFree plasmid prep kit derived DNA with EcoRI and SmaI, pAAV-DIO-mCherry had more bands by gel electrophoresis than expected, suggesting remaining undigested DNA. For pAAV-DIO-Mkrm3, the digested fragments were the anticipated sizes. Sanger sequencing of pAAV-DIO-mCherry showed annealing and sequence verification of one out of two primers. All five primers for the pAAV-DIO-Mkrm3 plasmid annealed and verified the plasmid sequence.

Conclusion: This model of selective Mkrm3 overexpression will provide insights to the mechanism of action of Mkrm3 in the hypothalamus and its potential to induce hypogonadism. The results of this study that this novel pAAV-DIO-Mkrm3 plasmid has the appropriate coding sequence and is now ready to be packaged into an AAV for stereotaxic injection into the ARC of female postpubertal Kiss1-Cre mice.

TABLE OF CONTENTS

DEDICATION	iv
ACKNOWLEDGMENTS	v
ABSTRACT.....	vi
TABLE OF CONTENTS.....	viii
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS.....	xii
INTRODUCTION	1
The Hypothalamic-Pituitary-Gonadal Axis	1
The Regulation of GnRH Secretion by Kisspeptin	3
Minipuberty.....	7
Physiology of Puberty.....	9
Factors Involved in Pubertal Onset.....	10
Monogenic Etiologies of Central Precocious Puberty	10
Role of MKRN3 in Delayed Puberty	16
Monogenic Etiologies of Idiopathic Hypogonadotropic Hypogonadism	19
SPECIFIC AIMS AND HYPOTHESIS	20
METHODS AND MATERIALS.....	21
Generation of Adeno-Associated Viruses and Mouse Model.....	21
Streaking and Isolating Colonies	23

Generation of the pAAV-EF1a-DIO-Mkrm3-IRES-mCherry (pAAV-DIO-Mkrm3)	
Plasmid.....	24
Bacterial Transformation	26
Restriction Enzyme Digestion	26
Sanger Sequencing.....	28
Study Approval and Animal Care	30
Generation of the Kiss1Cre Mouse Line	30
RESULTS	32
Plasmid DNA Purifications.....	32
Restriction Enzyme Digestions	34
Sanger Sequencing.....	36
Genotyping.....	39
DISCUSSION	40
CONCLUSION.....	44
BIBLIOGRAPHY	45
CURRICULUM VITAE.....	59

LIST OF TABLES

Table 1. Forward and reverse primers used for Sanger sequencing	29
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LIST OF FIGURES

Figure 1. Schematic representation of the HPG axis in males and females.	2
Figure 2. Differential regulation of kisspeptin neurons in the rodent ARC and AVPV by sex steroids.....	4
Figure 3. The role of ARC kisspeptin-neurokinin B-dynorphin (KNDy) neurons in pulsatile GnRH secretion	6
Figure 4. Gonadotropin secretion throughout fetal development and childhood.	8
Figure 5. Schematic representation of MKRN3 Structure.....	13
Figure 6. Relative levels of <i>Mkfn3</i> in the ARC of female and male mice.....	15
Figure 7. Schematic representation of the overlapping genetic causes of pubertal disorders.....	18
Figure 8. Plasmid maps for pAAV-DIO-mCherry and pAAV-DIO-Mkfn3.	22
Figure 9. Approach to inducing selective <i>Mkfn3</i> overexpression in KNDy neurons.....	25
Figure 10. Breeding strategy.....	31
Figure 11. Streaked agar plates and plasmid DNA quantification.....	33
Figure 12. Standard and endotoxin-free prep restriction enzyme digestions of pAAV-DIO-mCherry and pAAV-DIO-Mkfn3.....	35
Figure 13. Sanger sequencing of pAAV-DIO-mCherry with primer annealing locations.	37
Figure 14. Sanger Sequencing of pAAV-DIO-Mkfn3 with primer annealing locations..	38
Figure 15. Genotyping results.....	39

LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AAV-DIO-mCherry	AAV-EF1a-DIO-mCherry
AAV-DIO-Mkx3	AAV-EF1a-DIO-Mkx3-IRES-mCherry
ARC	Arcuate nucleus
AVPV	Anteroventral periventricular nucleus
BMI	Body mass index
CPP	Central precocious puberty
DIO	Double-floxed Inverted Open reading frame
DLK1	Delta-like 1 homolog
Dyn.....	Dynorphin
E2	Estradiol
ER- α	Estrogen receptor alpha
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HPG.....	Hypothalamic-pituitary-gonadal
IHH	Idiopathic hypogonadotropic hypogonadism
KO.....	Knockout
KOR	Kappa opioid receptor
KS	Kallman syndrome
LH	Luteinizing hormone
miRNA	MicroRNA

MKRN3.....	Makorin ring finger protein 3
NK3R.....	Neurokinin B receptor
NKB.....	Neurokinin B
PMV.....	Ventral premammillary nucleus
POA.....	Preoptic area
PWS.....	Prader-Willi Syndrome
RT.....	Room temperature
WT.....	Wild type

INTRODUCTION

The Hypothalamic-Pituitary-Gonadal Axis

The hypothalamus works in conjunction with the pituitary gland to maintain homeostasis in the body. The hypothalamic-pituitary-gonadal (HPG) axis regulates puberty and reproduction (Figure 1; Abreu et al., 2015). Neurons in the hypothalamus are involved in endocrine, autonomic, and behavioral responses to respond to various stimuli. Hormones such as gonadal steroids and glucocorticoids can negatively and positively regulate hypothalamic neurons (Douglas et al., 2018).

The hypothalamus releases gonadotropin-releasing hormone (GnRH), into the anterior pituitary gland, which then activates the release of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) into the bloodstream (Rawindraraj et al., 2023). LH and FSH act on the testes and ovaries to release steroid hormones, predominantly testosterone and estrogen, respectively (Ortiz et al., 2022).

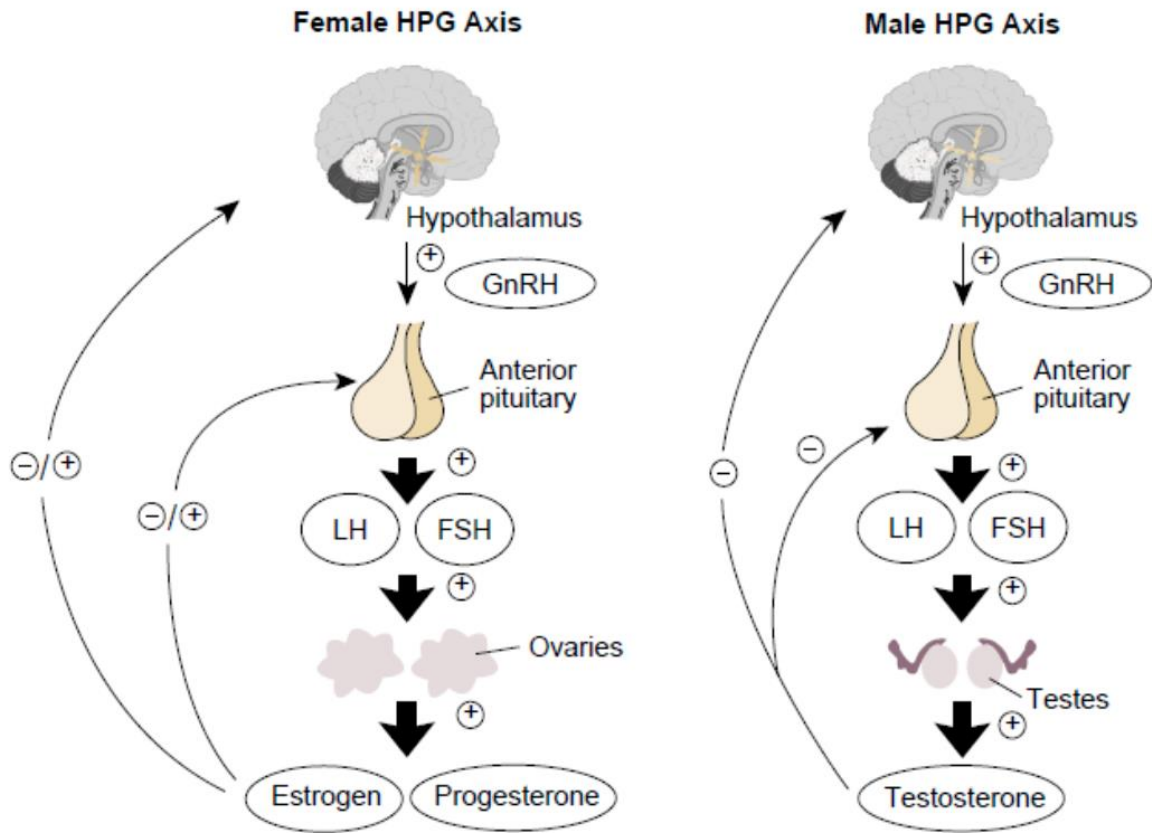


Figure 1. Schematic representation of the HPG axis in males and females.

The hypothalamus releases GnRH in a pulsatile manner. GnRH enters the pituitary through the hypophyseal portal system. Once in the pituitary gland, GnRH stimulates the production and release of the gonadotropins, LH and FSH, which move through the bloodstream and stimulate the production of sex steroids (Kong et al., 2014).

The Regulation of GnRH Secretion by Kisspeptin

The regulation of GnRH by kisspeptin is largely informed by rodent models. Upstream of GnRH, kisspeptin is a neuropeptide encoded by the rodent *Kiss1* gene and released by Kiss1 neurons, which are primarily localized in the rodent hypothalamic arcuate nucleus (ARC) and anteroventral periventricular (AVPV) nuclei. Steroid receptors in the ARC and AVPV send projections to the preoptic area of the brain, thereby communicating with GnRH neurons (Smith et al., 2005). However, GnRH neurons are not regulated directly by sex steroids because they lack androgen and estrogen receptors. It has been demonstrated that ARC Kiss1 neurons regulate the secretion of GnRH by exerting a negative feedback action through the binding of estradiol (E2) to the estrogen receptor, ER- α . Through this mechanism, Kiss1 neurons in the ARC regulate the tonic release of GnRH. In contrast, Kiss1 neurons in the AVPV drive the female LH surge before ovulation in response to the binding of E2 via positive feedback (Figure 2; Talbi & Navarro, 2020). Rodent Kiss1 neurons in the AVPV follow a sexually dimorphic distribution, with females having a greater density of Kiss1 neurons in the AVPV compared to males, which is nearly absent. This dimorphism leads to a greater amount of kisspeptin in the AVPV of females and is thought to play a role in the LH surge in the menstrual cycle (Wang & Moenter, 2019; Pereira et al., 2023).

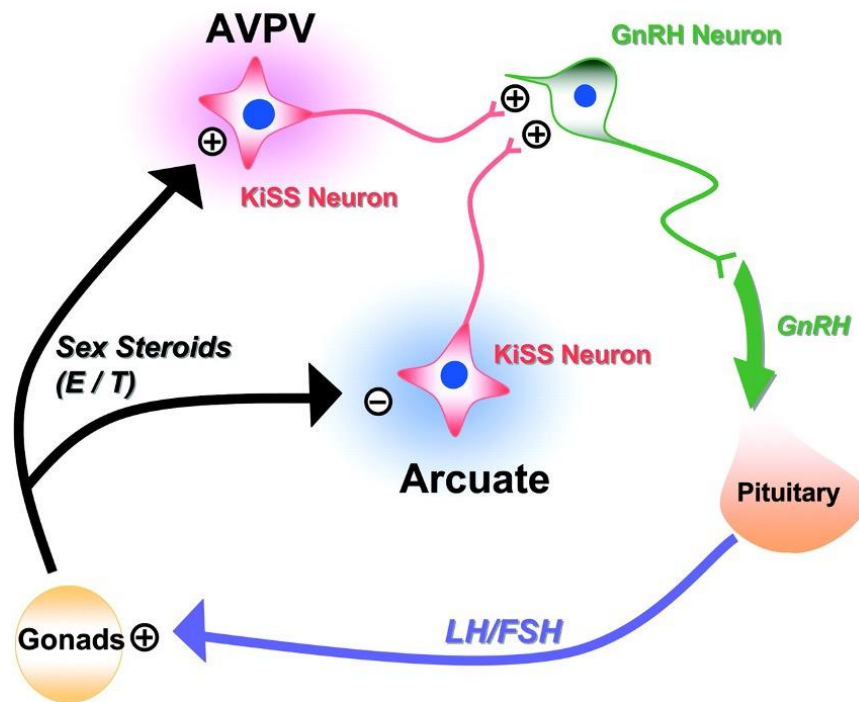


Figure 2. Differential regulation of kisspeptin neurons in the rodent ARC and AVPV by sex steroids.

Kiss1 neurons in the ARC and AVPV are regulated by the release of sex steroids from the gonads. In males, testosterone exhibits negative feedback on ARC Kiss1 neurons. In females, AVPV neurons are stimulated by the binding of E2 whereas ARC neurons are inhibited. This, in turn, regulates the tonic release of GnRH secretion (Dungan et al., 2006).

KNDy neurons are a specialized population of Kiss1 neurons in the ARC that co-express the neuropeptides kisspeptin, neurokinin B (NKB) and dynorphin (Dyn; Talbi and Navarro, 2020). NKB is a tachykinin that binds preferentially to its receptor, NK3R, which is encoded by the rodent genes, *Tac2* and *Tacr3*, respectively. Additionally, NK3R is expressed in GnRH neurons (Kauffman et al., 2009). Dyn is an opioid peptide expressed endogenously and binds to the κ -opioid receptor. NKB has been demonstrated to be a stimulator of kisspeptin secretion, while Dyn is an inhibitor (Navarro et al., 2013). In the comodulation of KNDy neurons via both NKB and Dyn, kisspeptin is released in an oscillatory fashion which leads to the same pulsatile pattern of release of GnRH (Figure 3; Navarro et al., 2009; Wakabayashi et al., 2010).

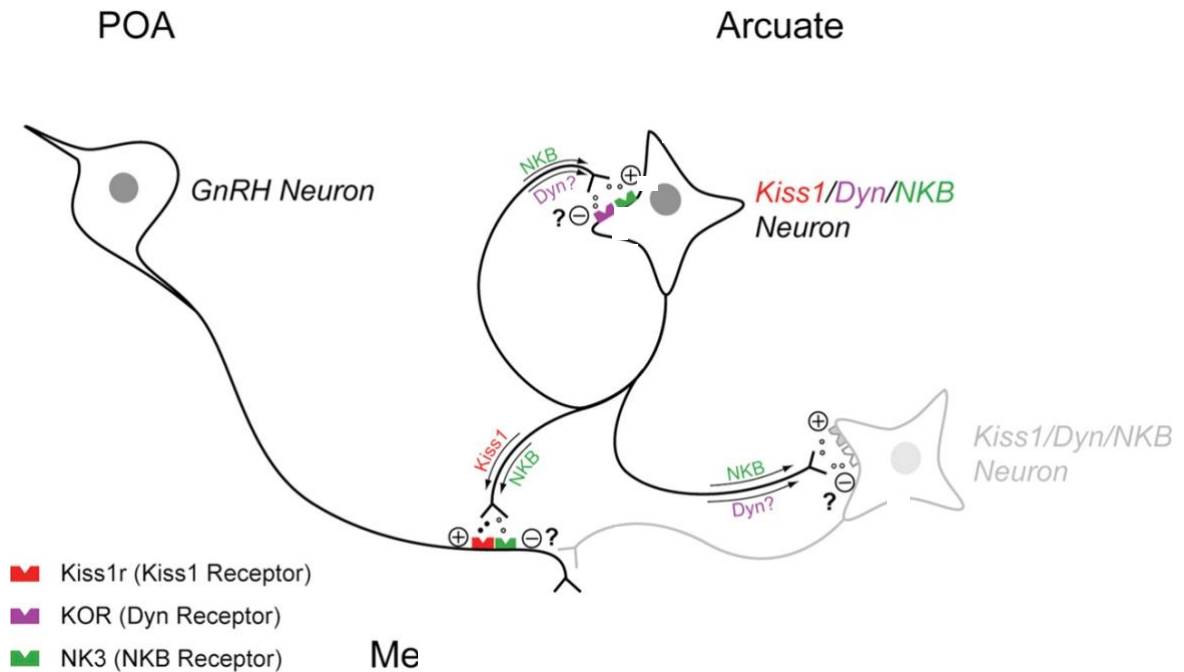


Figure 3. The role of ARC kisspeptin-neurokinin B-dynorphin (KNDy) neurons in pulsatile GnRH secretion. This schematic depicts the positive and negative feedback exerted by the products of KNDy neuron stimulation. The release of NKB and its interaction with its receptor, NK3R, lead to a stimulatory effect on kisspeptin secretion while dynorphin's interaction with its receptor, KOR, leads to an inhibitory effect. ARC KNDy neurons act on the cell body and axons of GnRH neurons and AVPV Kiss1 neurons project to the cell bodies located in the preoptic area (POA). Pulsatile kisspeptin release results in pulsatile GnRH release into the portal circulation. Adapted from Navarro et al., 2009.

Minipuberty

During neonatal development, the HPG axis undergoes two transient activations known as minipuberty (Figure 4). The fetal minipuberty occurs between the 10th and 24th week (Becker & Hesse, 2020). The levels of LH and FSH begin to rise at week 10 of gestation, peaking around 20 weeks. Rising levels of placental E2 lead to the suppression of LH and FSH secretion and at birth, serum levels of LH and FSH are undetectable (Renault et al., 2020). After birth, the removal of placental hormones from neonatal circulation results in the reactivation of the HPG axis which marks the beginning of the postnatal minipuberty (e.g. minipuberty of infancy). Due to the lack of negative feedback centrally, levels of LH and FSH increase in circulation (Lucaccioni et al., 2020). The levels of serum gonadotropins reach pubertal levels, but infants typically do not develop secondary sex characteristics.

It has been suggested that the absence of a minipuberty can be a marker for certain pubertal disorders. The diagnosis of various pubertal disorders (e.g. precocious or delayed puberty) presents a challenge because there are often no clinical signs at a young age that a child will have either early or delayed puberty in the future. For example, in idiopathic hypogonadotropic hypogonadism (IHH), some male patients with microphallus and cryptorchidism are described not to have experienced minipuberty, which is a crucial event in genital development (Swee & Quinton, 2019).

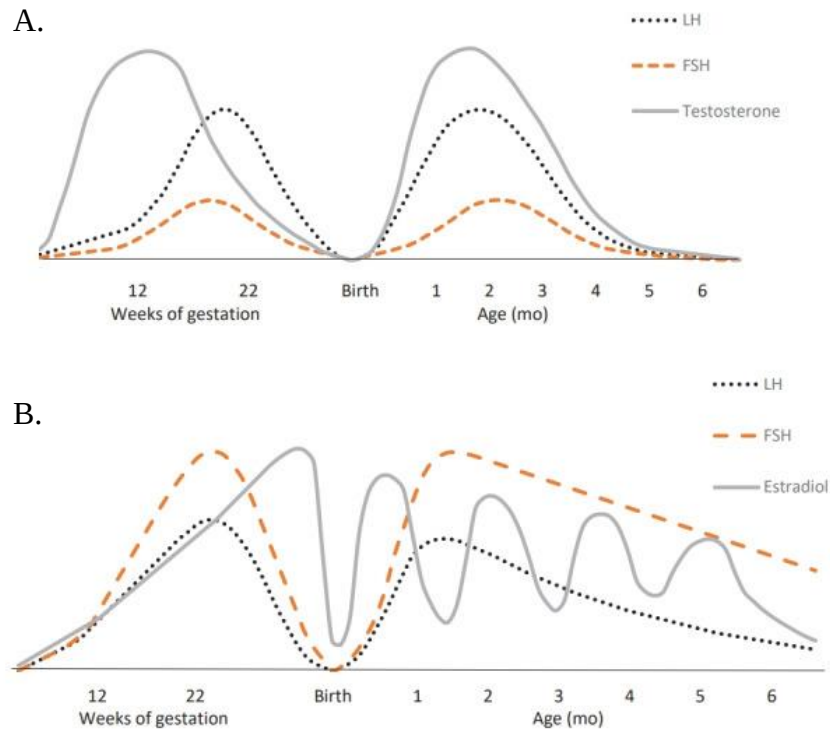


Figure 4. Gonadotropin secretion throughout fetal development and childhood.

In boys (A), during the first trimester in utero, human chorionic gonadotropin (hCG) stimulates the fetal production of testosterone. After the 9th week, testosterone synthesis is driven by LH, reaching peak levels by week 14 and thereafter decreasing to nearly undetectable levels by birth. In girls (B), in utero, LH, FSH, and E2 increase and peak at mid-gestation, and decrease significantly by birth. After birth, in both sexes, LH and FSH rise again marking the second activation of the HPG axis which is then quiescent until puberty (Renault et al., 2020).

Physiology of Puberty

The reactivation of the HPG axis at the onset of puberty leads to the development of secondary sex characteristics due to the increased levels of E2 and testosterone in the body. In the United States, in girls, this presents physically with breast development, occurring between 8 and 13 years old, with menarche (e.g. the first menstrual cycle) occurring on average 2 years later. During the female puberty, fat mass increases and an increased distribution of adipose tissue is seen around the hips and buttocks. In males, testicular enlargement occurs on average between 9 and 14 years old, alongside an increase in body hair growth and voice deepening as well as phallic enlargement. In both boys and girls, lean body mass increases as well as skeletal growth, characterized as a pubertal growth spurt (Wheeler, 1991). Pubertal development is characterized by Tanner staging, whose scaling is based on breast development in girls and external genitalia in boys, and pubic hair for both. The initial presence of breast buds in girls represents Tanner stage II, for example, which indicates the beginning of puberty. In males, a testicular volume of 4 mL or greater also indicates Tanner stage II and the onset of puberty (Emmanuel & Bokor, 2022; Roberts & Kaiser, 2020).

In females, LH acts on theca cells in the ovary and stimulates the synthesis of androgen precursors. FSH stimulates synthesis of aromatase, which aromatizes androgen precursors, producing primarily E2. FSH acts on its receptors in the granulosa cells to cause the development of an ovarian follicle and synthesis of E2. In males, LH acts on Leydig cells which leads to the production of testosterone. FSH acts on the Sertoli cells and stimulates spermatogenesis (Melmed et al., 2020; Shah et al., 2021).

Factors Involved in Pubertal Onset

There are various factors involved in the onset of puberty, including metabolic, environmental, and genetic factors. Metabolism is a key regulator in the onset of puberty. When negative energy balance occurs, physiological processes that are energetically costly, such as puberty, are halted. Conversely, obesity in girls has been associated with earlier puberty. The effect of body mass index (BMI) on male pubertal timing remains debated, but studies suggest an inverse relationship between BMI and pubertal onset as well (Sandhu et al., 2006; Busch et al., 2019). Recently, the focus has been placed on the genetic factors involved in pubertal onset. Genome-wide association studies of girls found that single-nucleotide polymorphisms in *Makorin ring finger protein 3* (*MKRN3*) and *Delta-like homolog 1* (*DLK1*) were associated with the age of menarche. Additionally, loss-of-function mutations in *MKRN3* have been identified in patients with central precocious puberty (CPP), e.g., a pathologically early onset of puberty (Valaderes et al., 2019).

Monogenic Etiologies of Central Precocious Puberty

Precocious puberty is classically defined as the onset of breast development before the age of 8 years in girls and testicular enlargement of 4 mL or greater before the age of 9 years in boys (e.g. 2 standard deviations earlier than the mean for each population). Precocious puberty can be classified as central or peripheral, or GnRH-dependent versus independent, respectively. CPP is due to premature activation of the HPG axis, leading to increased GnRH secretion at an earlier age. CPP affects girls more commonly than boys (Roberts & Kaiser, 2020).

Idiopathic CPP is more common in girls, historically constituting 90% of the diagnosed cases in girls however monogenic etiologies are being increasingly recognized as a cause. In contrast, around 50% of cases in boys have an identifiable organic cause. The diagnosis of CPP must be informed by a detailed history, physical exam, and bloodwork. A hormonal profile can also be tested to confirm central puberty. In addition to secondary sex characteristics, children with CPP will likely exhibit an increased growth velocity and advanced bone age compared to their peers, but an attenuated adult height. In young patients diagnosed with CPP, GnRH agonists are used to suspend puberty because they provide constant stimulus of GnRH to GnRH receptors on the pituitary, which results in downregulation of the pituitary-gonadal axis (Latronico et al., 2016).

Conversely, the much rarer peripheral precocious puberty does not occur as a result of early central HPG activation. Instead, peripheral precocious puberty can be due to the production of sex steroids from the gonads or adrenal glands, independent of central hormone production. Additional, exogenous steroid exposures and hormone-secreting tumors, as well as congenital diseases such as McCune-Albright Syndrome or congenital adrenal hyperplasia, can cause peripheral precocious puberty (Schoelwer & Eugster, 2017).

Mutations in certain genes have been identified in patients with CPP (Figure 5). These genes include *KISS1*, *KISS1R*, *MKRN3*, *DLK1*, and *MECP2* (Canton et al., 2023; Roberts & Kaiser, 2020). Loss-of-function mutations in *MKRN3* are the most common genetic cause of CPP. Pathologic variants in *MKRN3* were first identified in 2013 through

the use of whole exome sequencing in 15 families. This study found that, in five of the families, deleterious mutations in *MKRN3* was associated with a phenotype of central precocious puberty (Roberts & Kaiser 2020; Abreu et al., 2013).

The gene, *MKRN3*, which encodes a protein of the same name, is maternally imprinted, and paternally expressed. It is located in the Prader-Willi syndrome (PWS) region of chromosome 15q11.2 (Jong et al., 1999). PWS is a disorder characterized by hyperphagia and obesity, failure to thrive, and hypogonadism (Butler et al., 2019). The role of *MKRN3* in PWS is yet to be elucidated given these patients typically have hypogonadotropic hypogonadism, not CPP.

The structure of *MKRN3* includes a centrally located RING finger motif, two amino-terminal motifs, a pattern of Cys-His residues specific to the Makorin family, and a carboxy-terminal zinc finger motif (Figure 5). The protein structure, and recent research, support *MKRN3* is involved in RNA binding and E3 ubiquitin-ligase activity. (Abreu et al., 2015; Gray et al., 2000; Böhne et al., 2010). Ubiquitin ligase activity is the final part of the 3-part ubiquitination cascade, binding ubiquitin to target proteins and targeting them for degradation. There is evidence that defects in ubiquitin ligase function can lead to problems with cellular growth and development (Walma et al., 2022).

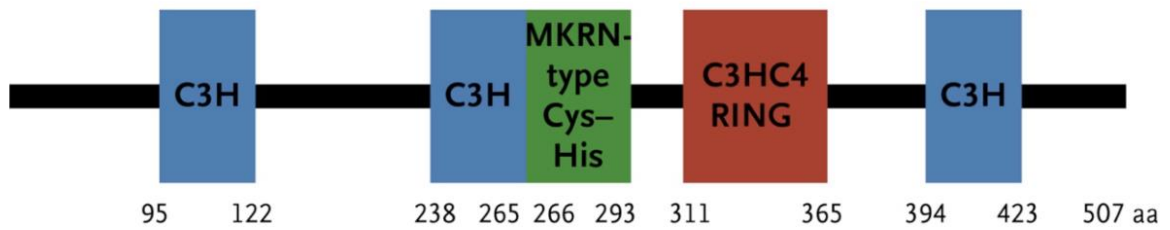


Figure 5. Schematic representation of MKRN3 Structure.

MKRN3 has three C3H domains (blue), a Cys-His domain (green), and one C3HC4 RING finger domain (red). The RING finger domain has been demonstrated to be important for the E3 ubiquitin ligase activity of MKRN3. The numbers correspond to the amino acid positions in the protein. Adapted from Abreu et al., 2013.

While the mechanism of action of MKRN3 is still being fully elucidated, there is strong evidence to suggest its role in the timing of puberty. MKRN3 is thought to function as a ‘brake’ in the initiation of puberty, inhibiting GnRH secretion. In neonatal and juvenile mice, *Mkfn3* mRNA levels in the hypothalamus are high in the juvenile period and declined rapidly before pubertal onset (Figure 6). Furthermore, declining serum levels of MKRN3 in humans before pubertal onset support the gene’s role as an inhibitor of pubertal expression, as well as the presence of loss-of-function mutations in patients with CPP (Roberts & Kaiser, 2020). It has been shown through mouse models that *Mkfn3* is expressed in GnRH neurons and ARC Kiss1 neurons, and through cellular models, can act as an inhibitor of *KISS1* and *TAC3* promoters (Abreu et al., 2015 & 2020). Naulé et al. (2023) differentiated hiPSC cells into ARC neurons and used CRISPR/Cas9 to induce a deficiency in *MKRN3*. They found that genes modulating neuronal plasticity in the ARC were downregulated in the *MKRN3*-deficient cells. Furthermore, *Mkfn3* knockout (KO) mice were generated from stem cells with a deletion of the *Mkfn3* gene and the breeding strategy utilized a paternally inherited *Mkfn3* deletion transmitted to the offspring (e.g. *Mkfn3*^{+/-}). In the KO mice, at postnatal days (PND) 10, 15, 20, and 25, *Kiss1* and *Tac2* mRNA expression were not increased in the ARC compared to control mice, but immunohistochemistry showed there was increased levels of NKB protein at PND 25 in the KO mice. This study also found that *Mkfn3* interacts with insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1), with increased levels of IGF2BP1 protein demonstrated in the ARC of *Mkfn3* KO mice.

IGF2BP1 is involved in embryogenesis, is highly expressed during murine development, and declines at birth.

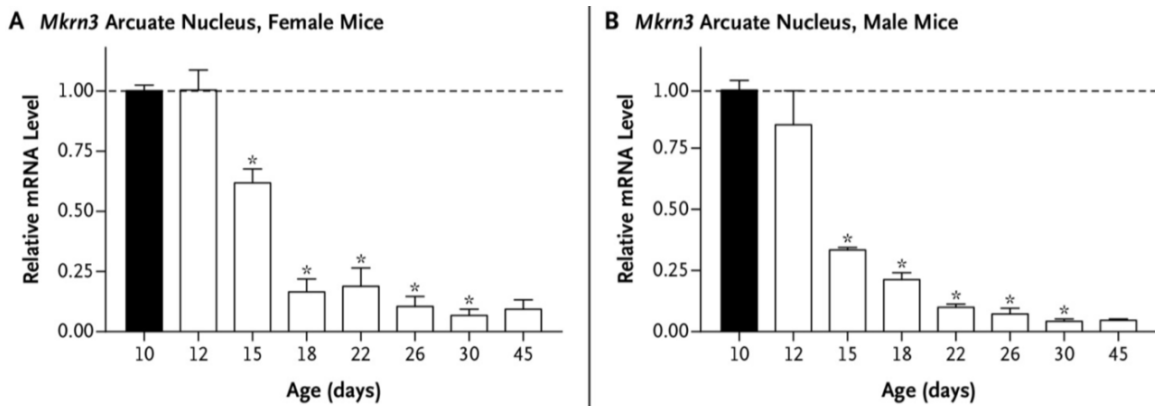


Figure 6. Relative levels of *Mkrn3* in the ARC of female and male mice.

In both female (A) and male (B) mice, *Mkrn3* mRNA levels decreased after postnatal days 10 and 12, when levels were the highest, in the ARC. Levels of *Mkrn3* mRNA continued to decrease until postnatal day 30. Abreu et al., 2013.

Role of MKRN3 in Delayed Puberty

Loss-of-function mutations in *MKRN3* have been identified as an important etiology of CPP. However, it is known that loss-of-function mutations in *KISS1* have been shown to cause IHH, while rare gain-of-function mutations in *KISS1* cause CPP (Figure 7; Topagalu et al., 2012; Silveira, 2010). Roberts et al. (2022) found that after bilateral intracerebroventricular injection of neonatal wild type (WT) mice with an adeno-associated virus (AAV) called AAV-*Mkfn3* to induce hypothalamic *Mkfn3* overexpression, females exhibited significantly delayed age of first estrus and delay in the age of vaginal opening, compared to the control AAV-EGFP injected mice. AAV-*Mkfn3* injected male mice that overexpress *Mkfn3* did not exhibit a pubertal phenotype significantly different from AAV-EGFP injected mice. Conversely, in the *Mkfn3* KO mouse model, females had significant advancement in the day of first estrus and, in males, advancement of preputial separation was observed but was not statistically significant (Naulé et al., 2023).

In vitro studies have also been performed to investigate the mechanism of action of MKRN3. Neuro-2a cells were transfected with a luciferase reporter stimulated by either the human *KISS1* or *TAC3* promotor, with or without a vector encoding *MKRN3*. In the empty vector, the transfection of both promotors increased luciferase expression, compared to a significant reduction in luciferase activity when *MKRN3* was co-transfected (Abreu et al., 2020). Assays using other promoters that play both stimulatory

and inhibitory roles in puberty showed that MKRN3 did not affect their expression, suggesting that MKRN3 may inhibit KNDy neurons to reduce kisspeptin and NKB in the ARC, leading to decreased GnRH secretion (Abreu et al., 2020). Cumulatively, this suggests that overexpression of *MKRN3* could lead to delayed puberty and/or hypogonadism through inhibition of KNDy neurons.

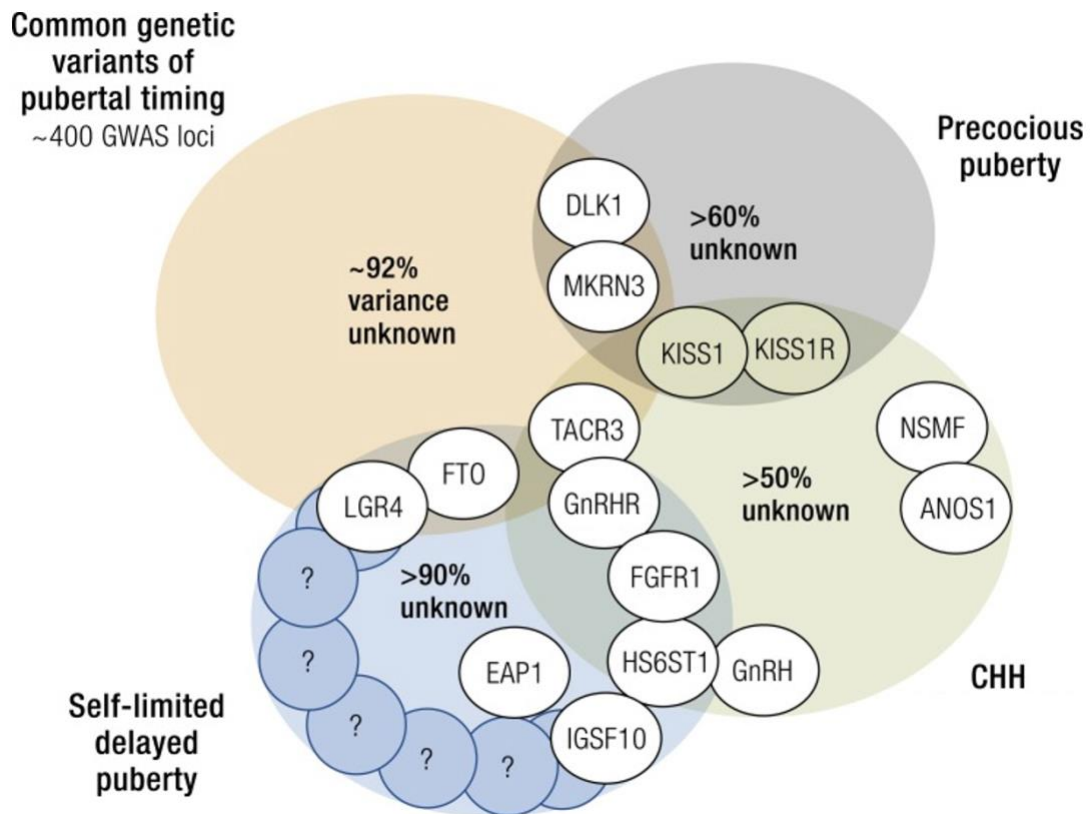


Figure 7. Schematic representation of the overlapping genetic causes of pubertal disorders.

There are many established genetic causes of disorders in pubertal timing, such as in IHH and precocious puberty. Self-limited delayed puberty (e.g. constitutional delay of growth and puberty) shares overlap of some genetic causes with congenital hypogonadotropic hypogonadism (CHH). Loss-of-function mutations in *KISS1* and *KISS1R* lead to CHH, while gain-of-function mutations lead to precocious puberty. Adapted from Howard and Dunkel, 2019.

Monogenic Etiologies of Idiopathic Hypogonadotropic Hypogonadism

IHH is a pubertal disorder characterized by the absence of onset of puberty or failure of progression of sexual development by 18 years of age. The most common cause of IHH is a lack of pulsatile GnRH secretion which leads to abnormally low levels of gonadotropins (Bianco & Kaiser 2009). In some cases, IHH is associated with anosmia (e.g. the loss of sense of smell) and is known as Kallman syndrome. GnRH neurons originate in the olfactory placode, which gives rise to the olfactory epithelium. They eventually migrate to the forebrain along with olfactory nerves. Kallman syndrome is caused in part by the failure of both olfactory and GnRH neurons to migrate to the forebrain (Cariboni & Maggi, 2006).

Kisspeptins bind to and activate the kisspeptin receptor GPR54, a G-protein coupled receptor, that elicits specific downstream effects upon ligand binding. Patients with deletions in the gene encoding GPR54 (e.g. *KISS1R*) were found to exhibit IHH. GPR54-deficient mice had similar results, thus recapitulating the phenotype observed in humans (Seminara et al., 2003).

There is overlap between the genes that encode olfactory and GnRH neurons, but not all patients with IHH have anosmia, suggesting multiple genetic pathways. Some examples of genes that are involved in Kallman syndrome include *KAL1*, *FGFR1*, *FGF8*, *PROK2*, and *PROKR2*, which encode proteins involved in neuronal migration. Some examples of genes involved in normosmic IHH include *GNRHR*, *KISS1*, *TAC3*, and *LEP* (Figure 5), which encode the proteins for the GnRH receptor, kisspeptin, NKB, and leptin, respectively (Bianco & Kaiser 2009).

SPECIFIC AIMS AND HYPOTHESIS

Based on prior, non-selective neuronal mouse models, the effects of widespread *Mkx3* overexpression in the central nervous system had inhibitory effects on KNDy neurons (Roberts et al., 2022). The overarching goal of this current project is to study the phenotype and mechanism of action when *Mkx3* is overexpressed in ARC KNDy neurons in postpubertal female Kiss1-Cre mice. We hypothesize that this model will lead to subsequent inhibition and quiescence of the HPG axis, manifested as full or partial central hypogonadism. This specific project aimed to generate novel AAVs that will overexpress *Mkx3* and subsequently be used for stereotaxic injection into the ARC of Kiss1-Cre female mice.

METHODS AND MATERIALS

Generation of Adeno-Associated Viruses and Mouse Model

Following the creation and validation of the sequences of two plasmids, pAAV-DIO-mCherry and pAAV-DIO-Mktn3, they will be packaged into novel AAVs (Mass Eye and Ear Gene Transfer Vector Core, Boston, MA). A recombinant AAV with the sequence AAV-EF1a-DIO-Mktn3-IRES-mCherry (abbreviated AAV-DIO-Mktn3) overexpressing *Mktn3* and a control virus with the sequence AAV-EF1a-DIO-mCherry (abbreviated AAV-DIO-mCherry) will be generated (Figure 8). This virus employs the use of a Double-Floxed Inverted Open (DIO) reading frame downstream of an EF1a promoter. Upon injection into the ARC of a Kiss1-Cre female mouse, the sequence in the 3' to 5' orientation (e.g. Mktn3-IRES-mCherry) will be flipped via homologous recombination and *Mktn3* will be selectively expressed in KNDy neurons (Figure 9). The internal ribosomal entry site (IRES) directs the ribosome to bind and allows for independent translation of Mktn3 and mCherry so that a fusion protein is not created (Renaud-Gabardos et al., 2015).

Figure 8. Plasmid maps for pAAV-DIO-mCherry and pAAV-DIO-Mkrn3.
The plasmid backbone pAAV-EF1-DIO-mCherry (A) was modified by inserting the IRES-Mkrn3 sequence via oligonucleotide synthesis to create pAAV-DIO-Mkrn3 (B). Figures generated by Snapgene

Streaking and Isolating Colonies

To prepare approximately 12 agar plates, 240 mL of sterile water was added to a 500 mL Erlenmeyer flask containing 8.88 g of Lennox Broth (LB; Fischer Scientific). The agar solution was autoclaved on a Liquid 45 cycle and allowed to cool for 30 minutes before adding 24 μ L of 100 mg/mL ampicillin. This yielded a concentration of 100 μ g/mL ampicillin. Each 10 cm agar plate was filled with the agar mixture and allowed to cool in the hood for 30 minutes, before being wrapped with parafilm and stored at 4°C.

The plasmid backbone pAAV-DIO-mCherry was obtained (Addgene, Watertown, Massachusetts) as an agar stab and streaked on agar plates, allowing it to incubate at 37°C overnight. Similarly, after receiving the modified plasmid, pAAV-DIO-Mkrn3 (GenScript; Figure 9), it was transformed into cells and streaked on separate agar plates containing 100 μ g/mL ampicillin and incubated at 37°C overnight.

Plasmid Preparation, DNA Extraction and Purification

For the inoculation, briefly, 1 L of LB was prepared using 25 grams of LB powder and 1 L of sterile water. The flask was covered with aluminum foil and held in place with autoclave tape and autoclaved on a Liquid 45 cycle. The broth was then stored at room temperature (RT) until use.

A starter culture was created by picking single colonies and inoculated in 5 mL of LB with 500 μ L ampicillin (100 μ L/mL). After 8 hours shaken at 250 rpm at 37°C, 500 μ L of starter culture was added to 250 mL or 500 mL LB for midi and maxi preps, respectively. The second culture was inoculated for 12-16 hours at 37°C at 250 rpm. After the second inoculation, glycerol stocks were collected. Briefly, in a 2.5 mL

centrifuge tube, 1 mL of the culture was added to 50% glycerol solution and stored at -80°C. The remaining bacteria culture was used for DNA purification using the Qiagen® Plasmid Midi Kit and the Qiagen EndoFree Maxi Kit per the manufacturer's protocol. The EndoFree Maxi kit was used to prepare samples to be sent for AAV packaging. DNA was eluted in Rnase free water for the non-EndoFree kits, and in TE Buffer for the EndoFree kits.

Generation of the pAAV-EF1a-DIO-Mkrn3-IRES-mCherry (pAAV-DIO-Mkrn3)

Plasmid

The purified DNA of the plasmid backbone pAAV-DIO-mCherry was quantified at 1,000 ng/μL in a total volume of 86 μL of Rnase free water. 10 μL was sent to a commercial vendor (GenScript, Piscataway, New Jersey) to insert *Mkrn3* and *IRES* coding sequences via oligonucleotide synthesis (Figure 9). The plasmid was returned as a lyophilized sample that was reconstituted in 20 μL of Rnase free water. After DNA purification (Qiagen plasmid prep kit) of both pAAV-DIO-mCherry and pAAV-DIO-Mkrn3 plasmids, the purified DNA was quantified using 2 uL on a NanoDrop® ND-1000 Spectrophotometer.

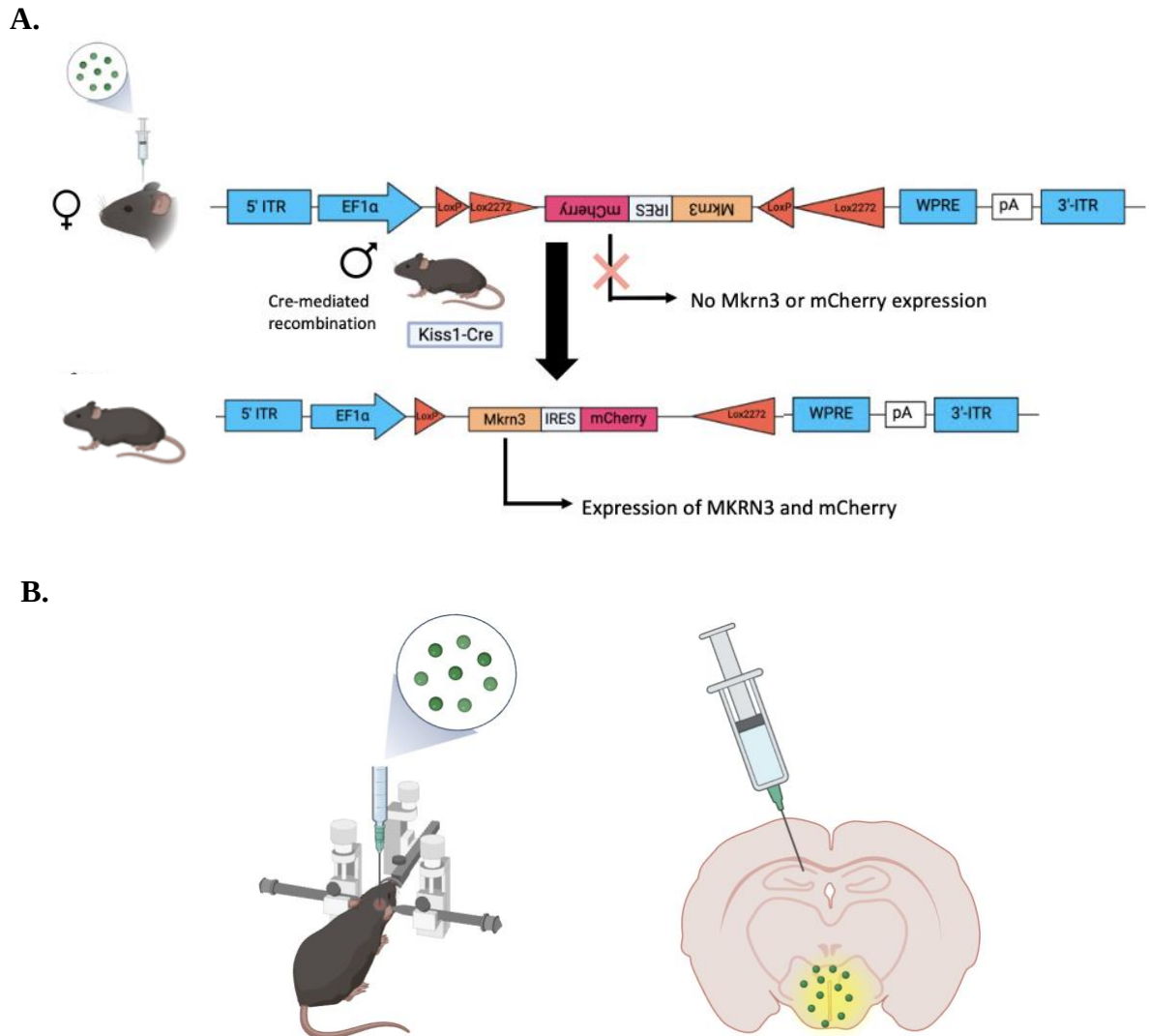


Figure 9. Approach to inducing selective *Mkrn3* overexpression in KNDy neurons. The DIO construct includes the mouse *Mkrn3* transgene, IRES, and *mCherry* coding sequences in the 3'→5' orientation (A). AAV-DIO-Mkrn3 will be stereotactically injected (B) into the ARC of Kiss1-Cre female mice resulting in selective *Mkrn3* overexpression in KNDy neurons. Figure generated with BioRender.

Bacterial Transformation

XL1-Blue Supercompetent Cells (Agilent) were removed from -80°C and allowed to thaw on ice for 30 minutes. Three agar plates containing 100 µg/1 mL of ampicillin were removed from 4°C and allowed to come to RT before incubating in the 37°C incubator. 1 µL of reconstituted pAAV-DIO-Mkrm3 plasmid DNA at a concentration of 0.2 µg DNA/µL in Rnase free water was mixed into 50 µL of competent cells in a 50 mL Falcon tube and mixed by gently flicking the bottom of the tube. The mixture was then incubated on ice for 30 minutes. The cells were then heat shocked by placing the bottom half of the falcon tube into a 42°C water bath for 45 seconds, and subsequently on ice for the proceeding 2 minutes. This process allowed for plasmid DNA uptake into the cells. 500 µL of LB was added to the bacteria. The mixture was incubated in a bacterial shaker at 37°C at 250 rpm for 45 minutes. The mixture was then streaked on each of the three agar plates using 10 µL, 50 µL, or 100 µL volume per plate. The plates were incubated overnight at 37°C.

Restriction Enzyme Digestion

Following transformation and DNA purification, the insertion of *Mkrm3* and *IRES* were confirmed using restriction enzyme digestion. In a restriction enzyme digestion, a target DNA molecule is incubated with a restriction enzyme that will cleave at specific sites on the DNA strand. For the restriction enzyme digestions, we used BamHI, EcoRI and/or SmaI to cut pAAV-DIO-mCherry and pAAV-DIO-Mkrm3 purified plasmid DNA. We also included a control sample of plasmid DNA without restriction enzyme (e.g. uncut plasmid). The concentration of purified plasmid DNA per sample was 148.0 ng/µL

for pAAV-DIO-mCherry and 198.6 ng/uL for pAAV-DIO-Mkrm3. Each digestion contained the following: 4 uL plasmid DNA, 1 uL restriction enzyme BamHI (New England BioLabs), EcoRI (Promega, Madison WI, 12 units/ μ L) or SmaI (New England BioLabs, 20,000 units/ μ L), or EcoRI with SmaI enzymes combined, 5 μ L of restriction enzyme buffer 1X NEBuffer EcoRI/SSPI (New England BioLabs) for EcoRI, or 10X rCutSmart buffer (New England BioLabs) for BamHI, and 1XcutSmart buffer, for SmaI (New England BioLabs). Rnase free water was added until the final volume of the solution was 50 μ L. As a control, 4 μ L of each purified plasmid DNA was diluted with 46 μ L of Rnase free water.

The DNA was allowed to incubate for 3 hours at 37°C. Following the restriction enzyme digestion, 2 μ L of each restriction enzyme digestion mixture was combined with 6 μ L of Rnase free water and 2 μ L of 5x loading dye (Qiagen). The DNA was visualized using a 0.8% agarose gel with ethidium bromide and compared to a 1 kilobase (Kb) ladder (New England BioLabs). The gel was made by adding 0.8 grams of agarose low electroendosmosis powder (Hoefer) to 100 mL of 1X Tris base, Boric Acid, and EDTA (TBE) and heated until boiling for 2 minutes. The solution was allowed to cool for 10 minutes before adding 10 μ L of ethidium bromide, mixing until homogenous, and allowing to set in with a 12 lane comb in place. 10 μ L of each sample was pipetted into the well, submerged in TBE, and ran for 2 hours, in a Thermofisher EasyCast B2 Mini Gel Electrophoresis system at 110 volts.

Sanger Sequencing

Sanger sequencing was performed to verify plasmid sequences. Five areas of the plasmid were selected, nucleotide sequence blasted using the National Library of Medicine National Center for Biotechnology Information (NCBI) Primer-BLAST online tool, and custom primers were manufactured by Invitrogen (Table 1). 10 μ L of 370.2 ng/ μ L of pAAV-DIO-Mkrm3 purified plasmid DNA was combined with 8.3 uL Rnase water and 1.7 uL of each primer (5 forward primers and 5 reverse primers), 10 samples in total. Additionally, 10 μ L of 385.1 ng/ μ L of pAAV-DIO-mCherry purified plasmid DNA was combined with 8.3 uL Rnase water and 1.7 uL of each primer (2 forward primers and 2 reverse primers), 4 samples in total. Samples were sent for Sanger sequencing (Massachusetts General Hospital Center for Computational and Integrative Biology). The results were analyzed using Sequencher (Gene Codes, Ann Arbor, MI) software program.

Plasmid Region	Forward
1. EF1a and LoxP	5'-GTTGCGTCAGCAAACACAGT-3'
2. LoxP and Mkrr3	5'-TGGGTAAAGGCAAATAACTTCGT-3'
3. Mkrr3	5'-CTTACCGAGGTCGCATGGTC-3'
4. Mkrr3 and IRES	5'-ATGACCGTTGGCTCTTGCTT-3'
5. mCherry and LoxP	5'-CTGAAGGGCGAGATCAAGCA-3'
	Reverse
1. EF1a and LoxP	5'-GGCCTCAATGGGAGCTGTAG-3'
2. LoxP and Mkrr3	5'-TGCACTGCCCATGCAGATAA-3'
3. Mkrr3	5'-TTCAACGCAGGCCCTTCTAT-3'
4. Mkrr3 and IRES	5'-CACACCGGCCTTATTCCAAG-3'
5. mCherry and LoxP	5'-TAGTCCTCGTTGTGGGAGGT-3'

Table 1. Forward and reverse primers used for Sanger sequencing. Five areas of the plasmid were selected and custom primers were designed

Study Approval and Animal Care

The animal protocol has been approved by the Brigham and Women's Hospital Institutional Animal Care and Use Committee (IACUC) in the Center for Comparative Medicine. The mice were housed according to sex and bred under constant conditions including temperature (22–24°C), a 12 hour light/dark schedule, fed with a standard rodent diet and constant access to tap water.

Generation of the Kiss1-Cre Mouse Line

Heterozygous female Kiss1-Cre^{+/-} mice were originally generated by Dr. Carol Elias (Jackson Labs #023426; Cravo et al., 2010). In order to generate heterozygous Kiss1-Cre female mice for future studies of this project, C57BL/6J Kiss1-Cre^{-/-} females were bred with heterozygous Kiss1-Cre males^{+/-} to produce Kiss1-Cre^{+/-} offspring. (Kiss1-Cre^{-/-} will also result from this breeding). Ear clippings were collected and sent for genotyping (Transnetyx), where DNA was isolated and PCR was completed to evaluate for the presence of Kiss1 and Cre gene expression (Figure 10).

Male Kiss1-Cre^{+/-}

Female Kiss1-Cre^{-/-}

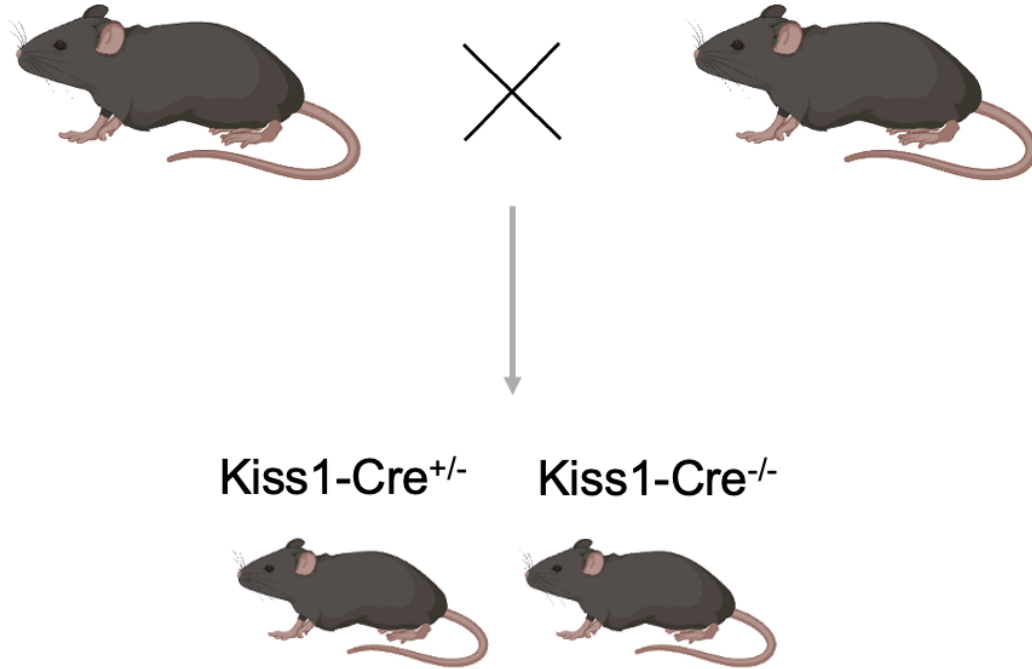


Figure 10. Breeding strategy.

Kiss1-Cre^{+/-} male mice were crossed with Kiss1-Cre^{-/-} females to produce Kiss1-Cre^{+/-} and Kiss1-Cre^{-/-} offspring. Figure generated with BioRender.

RESULTS

Plasmid DNA Purifications

Both plasmids were streaked on agar plates (Figures 11A and B) and inoculated overnight. A DNA extraction was conducted using Qiagen® Midi and EndoFree Maxi kits. The pAAV-DIO-mCherry and pAAV-DIO-Mkfn3 purifications were first executed using a Midi kit, and the purification done to prepare for AAV packaging via an EndoFree Maxi kit. The purpose of the endotoxin kit is to reduce the levels of endotoxins. Endotoxins are toxic substances found in the cell wall of Gram-negative bacteria which can result in sepsis in the mouse if present in AAVs that are then injected into mice (Kondratova et al., 2019). For the Midi plasmid prep, quantification showed that the DNA extracted from pAAV-DIO-mCherry had a concentration of 1123.4 ng/μL (in a volume of 78 μL; Figures 11C and D). DNA extracted from pAAV-DIO-Mkfn3 had a concentration of 2046.4 ng/μL (in a volume of 108 μL). The Endofree DNA extracted was 2113.8 ng/μL and the DNA extracted from pAAV-DIO-mCherry was 815.9 ng/μL (Figures 11E and F).

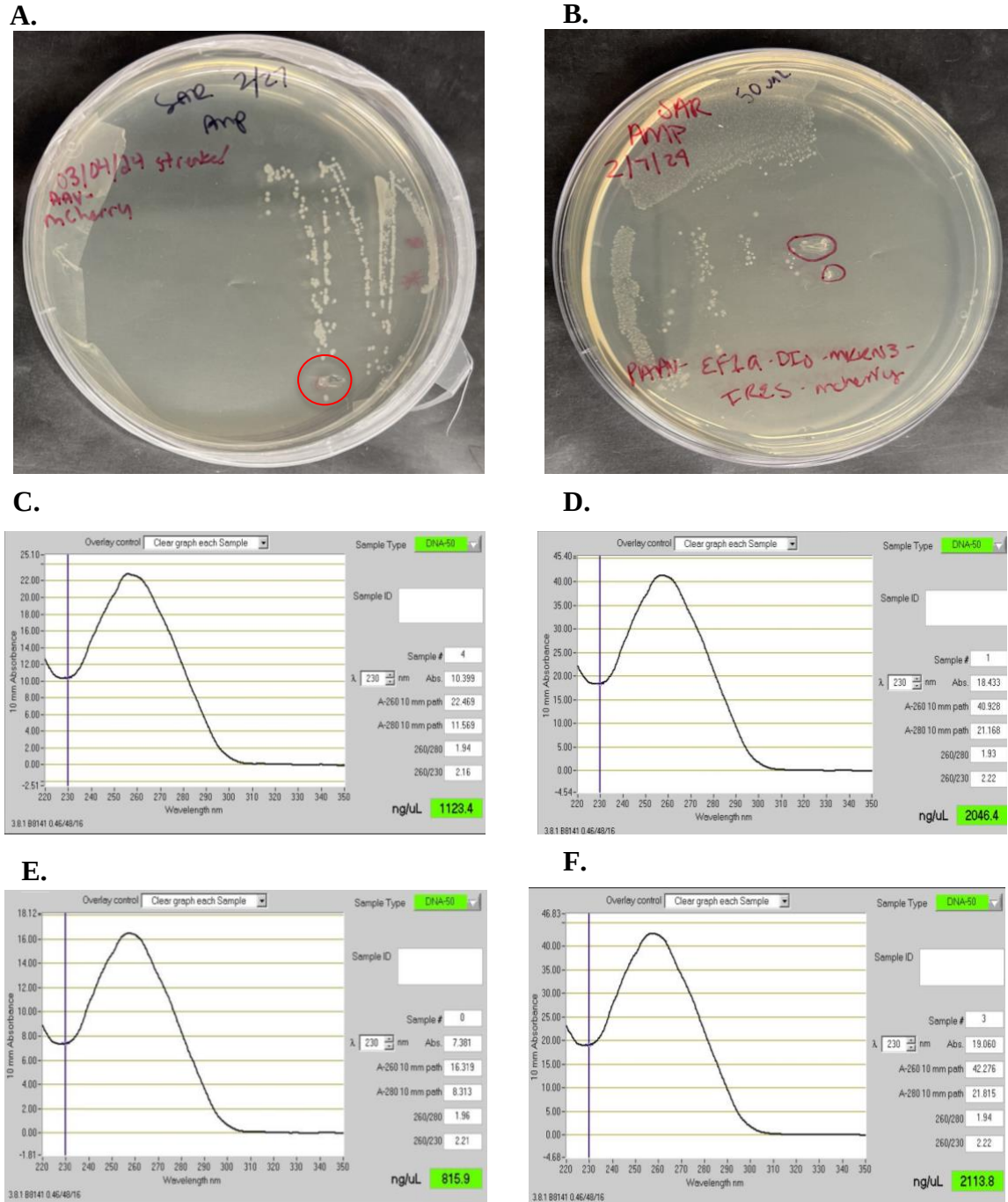


Figure 11. Streaked agar plates and plasmid DNA quantification. A. pAAV-DIO-mCherry colonies grown from an agar stab culture B. pAAV-DIO-MkRN3 colonies following transformation into supercompetent cells. The colonies that were picked for the plasmid prep kits are circled in red. C. DNA concentration of pAAV-DIO-mCherry D. DNA concentration of pAAV-DIO-MkRN3. E. EndoFree DNA concentration of pAAV-DIO-mCherry. F. EndoFree DNA concentration of pAAV-DIO-MkRN3.

Restriction Enzyme Digestions

Based on the pAAV-DIO-mCherry plasmid sequence (Figure 8A), we expected that EcoRI will produce a single 6 Kb fragment of linearized DNA. Similarly, BamHI will produce a single 6 Kb fragment of linearized DNA (Figure 12A). Based on the pAAV-DIO-Mkrn3 plasmid map (Figure 8B), we expected that EcoRI would produce 1.4 Kb and 7.0 Kb fragments. Furthermore, we expected that SmaI would produce 2.2 Kb, 2.6 Kb, and 3.6 Kb visible fragments (Figure 12B). SmaI has five cut sites, however, two of the fragments are too small to appreciate on the gel. Additionally, the combined digestion of EcoRI and SmaI should produce four bands, two from EcoRI and three from SmaI, at the same molecular weights as the separate digestions. The undigested plasmid showed only one band since no restriction enzyme was added. We repeated the digestions following the completion of a Qiagen Endofree Maxi kit (Figures 12C and D).

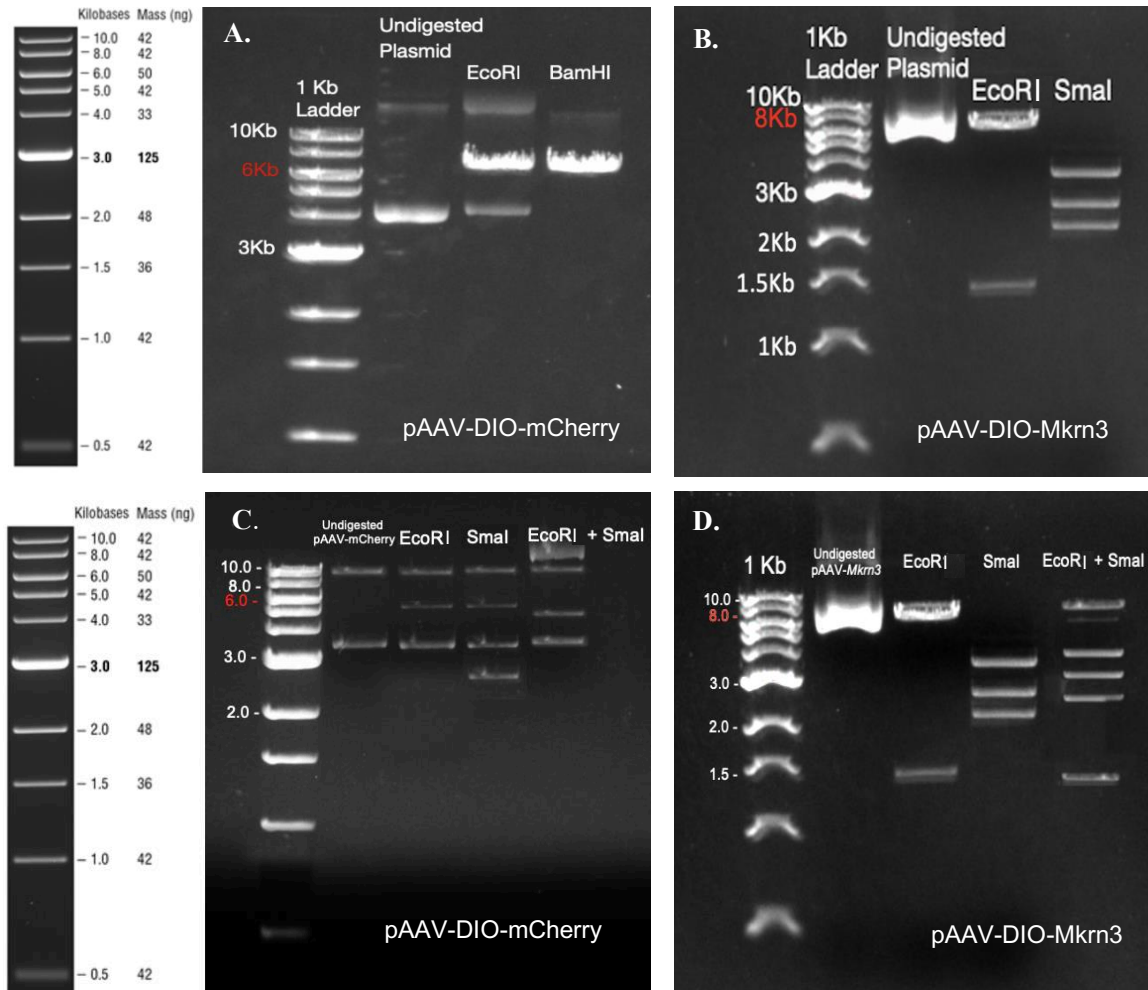


Figure 12. Standard and endotoxin-free prep restriction enzyme digestions of pAAV-DIO-mCherry and pAAV-DIO-Mkrn3. A. Standard prep restriction enzyme digestion of pAAV-DIO-mCherry. B. Standard prep restriction enzyme digestion of pAAV-DIO-Mkrn3. C. EndoFree prep restriction enzyme digestion of pAAV-DIO-mCherry. D. EndoFree prep restriction enzyme digestion of pAAV-DIO-Mkrn3. In pAAV-DIO-mCherry, EcoRI and BamHI created linearized fragments at approximately 6.0 Kb. In pAAV-DIO-Mkrn3, EcoRI created fragments at 1.4 Kb and 7.0 Kb. SmaI created visible fragments at approximately 2.2 Kb, 2.6 Kb, and 3.6 Kb. 1 Kb Ladder included for reference. Red text indicates predicted plasmid size.

Sanger Sequencing

To further validate the plasmid, both plasmids were sent for Sanger sequencing. Primers 1 and 5 were used for pAAV-DIO-mCherry (Table 1, Figures 13A-C). Primer 1 annealed and verified the sequence (Figure 13B) however primer 5 did not anneal despite being sent for sequencing twice (Figure 13C). For pAAV-DIO-Mkrn3, primers were designed to bind to the designated areas in the plasmid: the four LoxP sites, Mkrn3, IRES, and mCherry (Figures 14A). Primer 3 (13B, C) both annealed and verified the *Mkrn3* sequence.

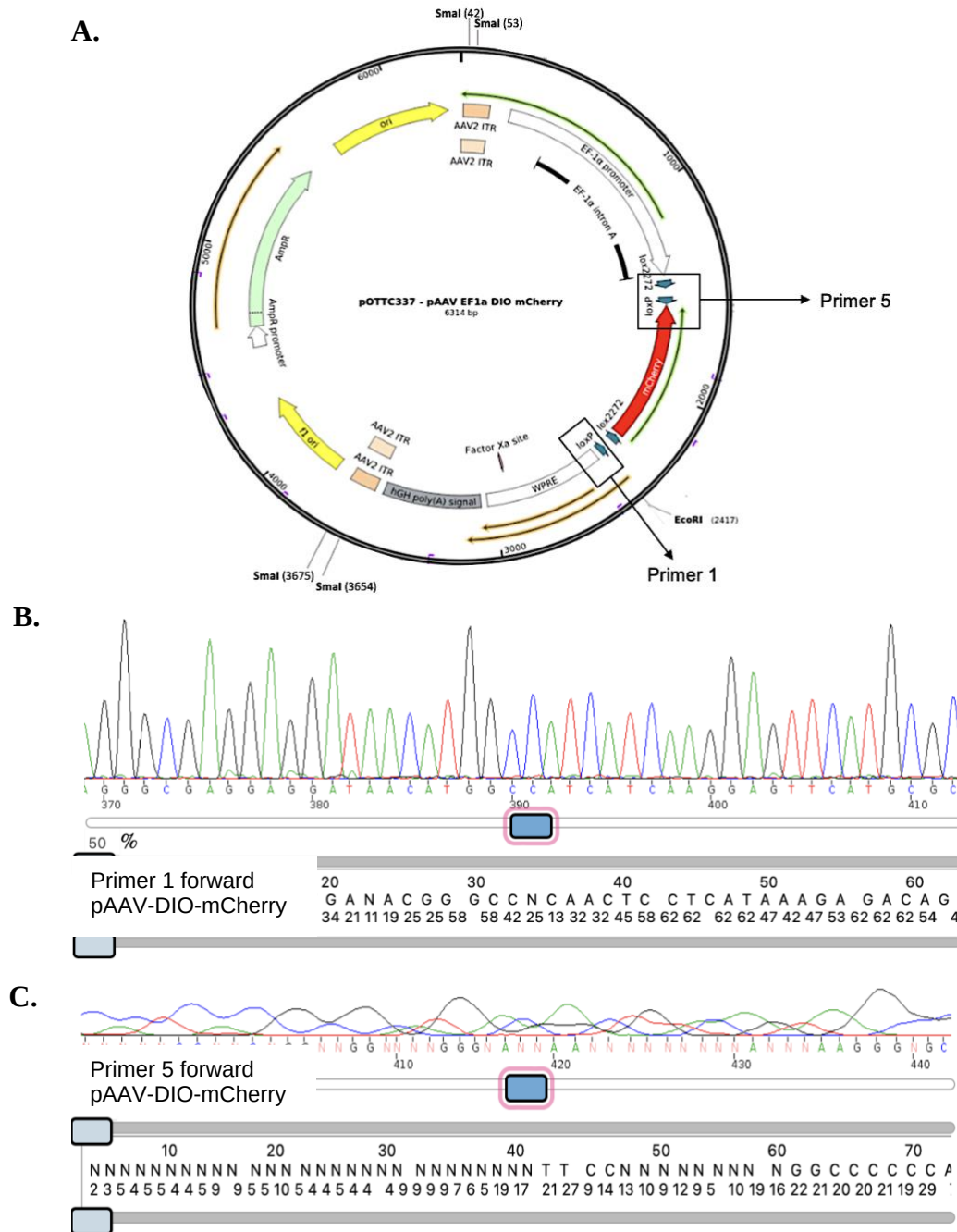


Figure 13. Sanger sequencing of pAAV-DIO-mCherry. Since this was prior to oligonucleotide synthesis, only primers 1 and 5 (A) were appropriate to use to verify the nucleotide sequence of pAAV-DIO-mCherry. Representative sequencing showed Primer 1 forward (B) annealed appropriately and verified the expected sequence but primer 5 forward (C) did not anneal. Plasmid map generated by Snapgene.

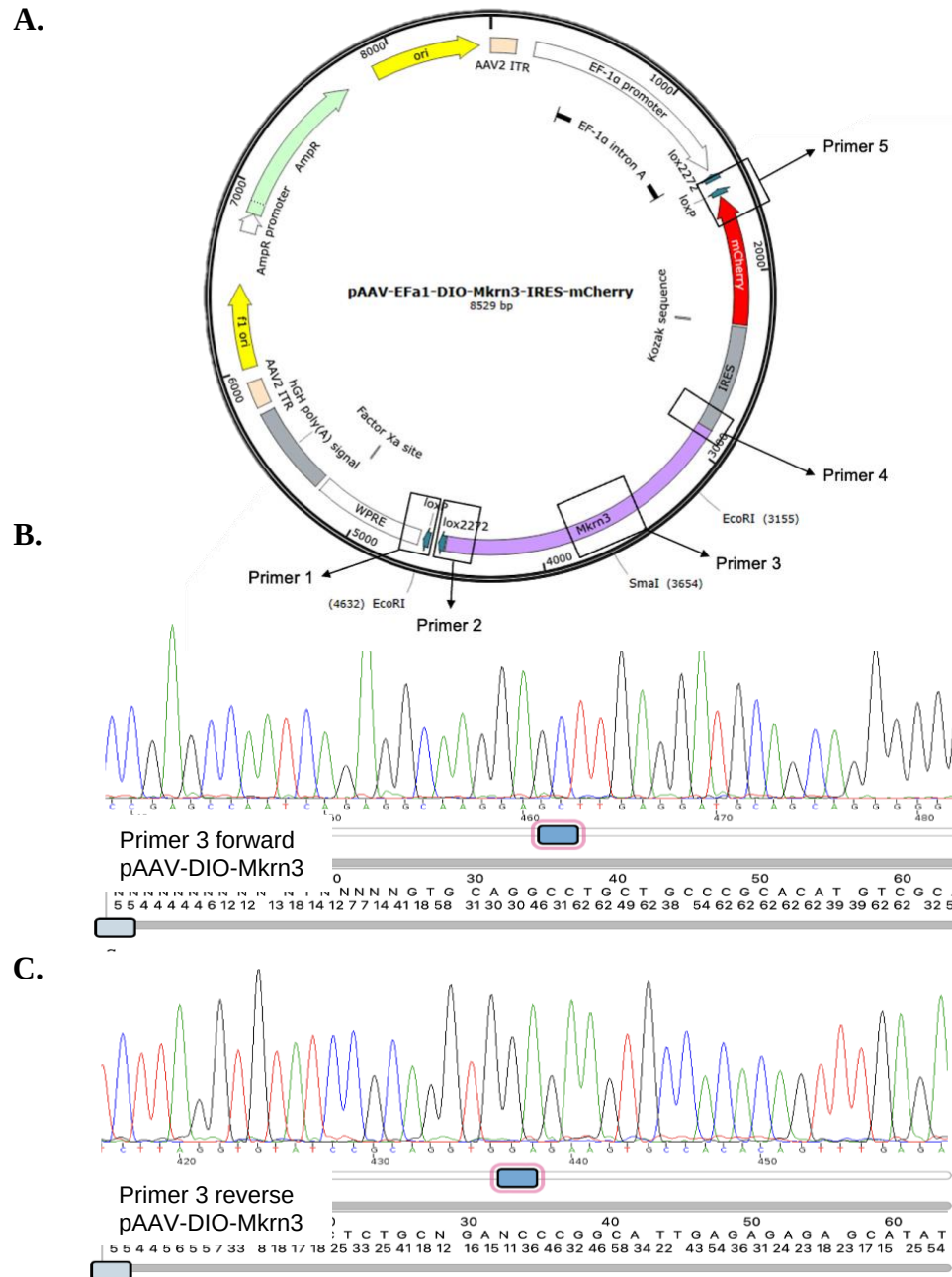


Figure 14. Sanger Sequencing of pAAV-DIO-Mkrn3. Custom primers were designed to confirm the nucleotide sequence of the plasmid after oligonucleotide synthesis (A). Representative sequencing of Primer 3 forward (B) and reverse (C) in the *Mkrn3* sequence shows the primer annealed and had the correct sequence. Plasmid map generated by Snapgene.

Genotyping

After crossing a Kiss1-Cre^{+/+} male mouse with a Kiss1-Cre^{-/-} female, the litter is anticipated to produce both heterozygote Kiss1-Cre^{+/+} and Kiss1-Cre^{-/-} mice. The genotyping also evaluates for Kiss1, however, this is an internal control. All animals are Kiss1^{+/+} on the genotyping, because their endogenous *Kiss1* expression is unaffected in this mouse model (Cravo et al., 2010). The results of the genotyping showed 5 Kiss1-Cre^{+/+} mice and 1 Kiss1-Cre^{-/-} mouse (Figure 15).

☐		Kiss1 CRE-Elias 	CRE	Kiss1-1 WT
Wellplate: T1814619				
☐	A1	F7801	+	+
☐	B1	F7802	+	+
☐	C1	M7803	+	+
☐	D1	M7804	+	+
☐	E1	M7805	+	+
☐	F1	M7807	-	+

Figure 15. Genotyping results of Kiss1-Cre mouse line. Kiss1-Cre^{-/-} females were crossed with Kiss1-Cre^{+/+} males to produce Kiss1-Cre^{+/+} heterozygotes and Kiss1-Cre^{-/-} control mice. All animals are Kiss1 positive because their endogenous *Kiss1* expression is unaffected.

DISCUSSION

Pubertal onset is marked by the reactivation of the HPG axis after a period of childhood quiescence. MKRN3 is involved in the onset of puberty and functions as a ‘brake,’ inhibiting the activation of GnRH secretion. In patients with CPP, loss-of-function mutations in *MKRN3* lead to premature activation of GnRH secretion and early onset of puberty (Abreu et al., 2013). A neonatal mouse model of *Mkfn3* overexpression in the mediobasal hypothalamus and POA demonstrated delayed puberty in female mice, indicating that *Mkfn3* overexpression may also have a role in delayed puberty and/or hypogonadism (Roberts et al., 2022). Based on this, we aimed to create a novel mouse model of selective *Mkfn3* overexpression in KNDy neurons to determine the role of *Mkfn3* in postpubertal mice.

MKRN3 is expressed in KNDy neurons, which are a specialized population of *Kiss1* neurons and prior studies have examined MKRN3’s mechanism of action in this important neuronal population. MKRN3 exerts its inhibitory effects by acting on KNDy neurons, such as through inhibiting *KISS1* and *TAC3* promoters which was observed in *in vitro* studies (Abreu et al., 2020). Naulé et al. (2023) showed that female *Mkfn3* KO mice showed a significant advancement in the day of first estrus compared to control mice. At PND 10, 15, 20, and 25, ARC mRNA levels of *Kiss1* and *Tac2* were measured in both KO and WT, and showed no significant difference between the two. However, at PND 25, an increase in NKB protein in the ARC was observed in *Mkfn3*-deficient mice. In a contrasting model, Roberts et al. (2022) in a neonatal mouse model of hypothalamic *Mkfn3* overexpression, showed an increase in the mRNA levels of *Kiss1* and *Tac2* in the

ARC at PND28, but decreased levels of kisspeptin and NKB protein. These surprising findings suggested that *Mkfn3* may also act to degrade the neuropeptides secreted by KNDy neurons directly or further downstream of KNDy neurons. The increased amount of *Kiss1* and *Tac2* mRNA observed in these studies may also have occurred as a result of compensatory upregulation if kisspeptin and NKB protein were degraded. However, further studies are needed to further elucidate the mechanism of action of *Mkfn3*. Overall, these data suggest that *Mkfn3* acts on KNDy neurons in the ARC, as supported by models of *Mkfn3* deficiency and overexpression.

Thus, we hypothesize that in this current mouse model, which aims to overexpress *Mkfn3* in KNDy neurons via stereotaxic injection into the ARC, female mice will have a postpubertal phenotype consistent with full or partial central hypogonadism. This phenotype is anticipated to be manifested as disrupted estrous cycles and potentially increased time in diestrus (the time between periods of estrous). We also anticipate that *Mkfn3* overexpression will lead decreased LH pulsatility.

Currently, our selective novel mouse model will serve to overexpress *Mkfn3* only in ARC KNDy neurons to a) specifically study the effects on kisspeptin neurons and b) to explore if there are only distinct developmental windows that *Mkfn3* can exert inhibitory effects on the neuroendocrine control of reproduction. To generate the selective AAV-injected mouse model, this project first focused on generating the plasmids with the appropriate constructs for AAV packaging. To create AAV-DIO-*Mkfn3*, pAAV-DIO-mCherry was modified to include *Mkfn3* and IRES, which were inserted via oligonucleotide synthesis. The results of this study focused on validation of the backbone

(e.g. AAV-DIO-mCherry) and experimental plasmid (AAV-DIO-Mkrm3). pAAV-DIO-mCherry will also undergo AAV packaging and will be utilized to generate stereotactically-injected control mice.

Prior to AAV packaging, an EndoFree plasmid DNA purification kit was required. Endotoxin is a lipopolysaccharide found in the outer membrane of Gram-negative bacteria. The presence of endotoxin can result in gram-negative sepsis in mice and can potentially lead to death (Kondratova et al., 2019). The EndoFree kit is to ensure that endotoxins will not cause a reaction in the mouse host after injection of AAV-DIO-Mkrm3 or AAV-DIO-mCherry.

In the pAAV-DIO-mCherry backbone, the restriction enzyme digestion with the EndoFree kit showed bands that were not predicted and validation of this plasmid is ongoing. The only difference in these sample was that the EndoFree protocol was conducted using glycerol stocks, whereas the other protocol was conducted using the agar stab from a commercial vendor. It is possible that a type of transformation took place in the glycerol stock that inhibited the restriction enzymes as the bands appear to be undigested DNA. However, it is also concerning that this plasmid could not fully validated by Sanger sequencing. Future directions will include considering transforming the pAAV-DIO-mCherry plasmid into supercompetent cells using the same heat shock protocol that was used for pAAV-DIO-Mkrm3. Currently, the plan is to obtain a new pAAV-DIO-mCherry agar stab and perform plasmid DNA purification and repeat the restriction enzyme digestion and Sanger sequencing.

The pAAV-DIO-Mkrn3 is validated and ready for AAV packaging. Custom primers were designed to confirm the coding sequence of WPRE, *Mkrn3*, *IRES*, the four *LoxP* sites, and mCherry. In the plasmid, the purpose of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) site is to reduce transcription readthrough, which is the failure of transcription to stop at designated stop sites (Higashimoto et al., 2007; Caldas et al., 2024). The purpose of the IRES sequence is to allow internal binding of the ribosome, which facilitates bicistronic gene expression – in this case, the expression of both *Mkrn3* and mCherry in one vector without being a fusion protein (Gabardos et al., 2015).

Once fully validated, both plasmids will be sent for AAV packaging. AAVs are small, single-stranded DNA parvoviruses that are commonly used in genetic research because they are non-pathogenic and cause less immunoreactivity than other vectors. AAVs have varying tropism based on their serotypes (Issa et al., 2023). It is important to choose a serotype that allows efficient gene transfer to target cell types. In our case, AAV9 will be utilized because it can cross the blood-brain barrier and be transduced in neuronal and non-neuronal cell types in the brain (Lin et al., 2020). This model employs the use of a DIO which includes a floxed *Mkrn3*-*IRES*-mCherry sequence in the 3'→5' orientation. At baseline, this DIO, also known as the FLEX, will not be expressed. This DIO flanks the transgene with two mutant loxP (e.g. lox2272) and two WT loxP sites, in opposite directions, which are unable to undergo recombination.

After inversion, one pair of loxP sites is cleaved out to prevent re-inversion (Xu and Zhu 2018). We chose to use this system because it is an efficient way to induce cell-

specific genetic overexpression of a target with minimal off-target expression. DIOs are also much smaller than other constructs, which allows for packaging into an AAV (Botterill et al., 2021).

Briefly, we are also employing the use of a CRISPR/Cas9 transgenic mouse model of *Mkfn3* overexpression, via the ROSA26 locus. This type of mouse model allows us to observe the effects of overexpression which will be able to be observed starting neonatally, whereas in our current model, we will observe changes postpubertally. This will also affect all *Kiss1* neurons in the brain, not just ARC KNDy neurons.

In this current model, if a hypogonadal phenotype is not observed, this would still be a topic of interest since it suggests that *Kiss1* neurons may not be the site of *Mkfn3* action. However, such a result could also be observed if AAV-DIO-*Mkfn3* was injected outside of the developmental window where *Mkfn3* can exert inhibitory effects on the HPG axis.

CONCLUSION

Ultimately, the findings of this greater study if central hypogonadism is induced with the *Mkfn3*-overexpressing AAV, could be used as a new therapeutic target. Current findings provide evidence for the action of *Mkfn3* on NKB, which could indicate potential for new therapeutics for pubertal disorders such as CPP or other disorders where inhibition of the central neuroendocrine control of reproduction is desired. Currently, more research is needed to further understand precisely where *Mkfn3* acts to exert its effects.

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CURRICULUM VITAE

