UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Novel Gonadotropin-Releasing Hormone 1 (*Gnrh1*) Enhancer-Derived Noncoding RNA in the Regulation of *Gnrh1* Gene Expression

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Polly Pu Huang

Committee in charge:

Professor Pamela L. Mellon, Chair
Professor Alexander S. Kauffman
Professor Nicholas J. G. Webster
Professor Miles F. Wilkinson
Professor Eugene W. Yeo

2016

The Dissertation of Polly Pu Huang is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

Chair

University of California, San Diego

2016

DEDICATION

I dedicate my dissertation to my best friend and the love of my life, Steffan McMurrin, for his support, encouragement, guidance, empathy, and unwavering love.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3C	Chromosome conformation capture
Act D	Actinomycin D
BGH	Bovine growth hormone
bp	Base pair
ChIP	Chromatin immunoprecipitation
ChIRP	Chromatin isolation by RNA purification
CMV	Cytomegalovirus
DMSO	Dimethyl sulfoxide
eRNA	Enhancer RNA
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GnRH-E	*Gnrh1* enhancer
GREKO	GnRH enhancer knockout
H2afz	Histone 2 A.Z
HPG	Hypothalamus-pituitary-gonad
IHH	Idiopathic Hypogonadotropic Hypogonadism
kb	Kilobases
LH	Luteinizing hormone
ICV	Intracerebroventricular
lincRNA	Long intergenic noncoding RNA
lncRNA	Long noncoding RNA
ncRNA	Noncoding RNA
NKB	Neurokinin B
nt	Nucleotide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PolyA	Polyadenylation
Ppia	Peptidylprolyl isomerase A
QPCR	Quantitative polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RIP	RNA immunoprecipitation
RNA Pol II	RNA Polymerase II
RSV	Rous sarcoma virus
RT	Reverse transcriptase or reverse transcription
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SV40	Simian virus 40
TF	Transcription factor
TK	Thymidine kinase
TPA	12-O-tetradecanoyl-phorbol-12-acetate
TSS	Transcription start site

ACKNOWLEDGEMENTS

I would like to acknowledge and thank Professor Pamela L. Mellon for her continued support and guidance throughout my graduate studies. Her mentorship has been invaluable to both my research and to myself as a scientist. I would like to thank the members of my thesis committee: Professors Alexander S. Kauffman, Nicholas J. G. Webster, Miles F. Wilkinson, and Eugene W. Yeo, for their advice, guidance, and feedback. I would like to thank the Biomedical Sciences Graduate Program for providing me the opportunity and the community in which to conduct my research.

I would like to give special thanks to Dr. Anita K. Iyer for early contributions to my Dissertation project, and for training, mentoring, and supervising my work in the lab during my rotation. I also give special thanks to Dr. Melissa J. Brayman for her early contributions and RNA sample preparation for RNA sequencing experiments. I would like to thank Dr. Nicholas J. G. Webster for RNA sequencing analysis and bioinformatics assistance. I would like to thank Chelsea Painter for her assistance with transfection experiments, which contributed to parts of the research in this Dissertation. In addition, I would like give special thanks to Liza Brusman, whose work has contributed to parts of this Dissertation, and who has been a tireless assistant through the final stages of my project.

I would like to thank all of the members Mellon Laboratory, both past and present, for all of their technical help and support. I would like to give special thanks to Dr. Christine Glidewell-Kenney and Dr. Hanne Hoffmann for their technical support in cloning and transfections. I appreciate the constant advice and support from the members of the Department of Reproductive Medicine, including members of the Kauffman Lab, Lawson Lab, Thackray Lab, and Church Lab.

The work in this Dissertation has been supported by National Institutes of Health (NIH) Grants R01 DK044838, R01 HD072754, and R01 HD082567. It was also supported by National Institute of Child Health and Human Development/NIH through a cooperative agreement (U54 HD012303) as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research. Polly Huang was supported by NIH T32 DK007541.

The work in this Dissertation has been submitted to the Public Library of Science One (PLoS One). The following co-authors of the submitted manuscript gave their permission to include their contributions in this Dissertation: Brusman, Liza E., Iyer, Anita K., Webster, Nicholas J. G., and Mellon, Pamela L.

VITA

University of California, San Diego

2009 Bachelor of Science, Human Biology

2010 Master of Science, Biology

2016 Doctor of Philosophy, Biomedical Sciences

PUBLICATIONS

P. P. Huang, L. E. Brusman, A. K. Iyer, and P. L. Mellon (2016). “A novel gonadotropin-releasing hormone 1 (*Gnrh1*) enhancer-derived noncoding RNA regulates *Gnrh1* gene expression in GnRH cell models.” PLoS One. Submitted.

Marino, M., P. P. Huang, S. Malkmus, E. Robertshaw, E. A. Mac, Y. Shatterman, and T. L. Yaksh (2012). "Development and validation of an automated system for detection and assessment of scratching in the rodent." J Neurosci Methods **211**(1): 1-10.

P. P. Huang, I. Khan, M. S. Suhail, S. Malkmus, and T. L. Yaksh (2011). "Spinal botulinum neurotoxin B: effects on afferent transmitter release and nociceptive processing." PLoS One **6**(4): e19126.

ABSTRACT OF THE DISSERTATION

A Novel Gonadotropin-Releasing Hormone 1 (*Gnrh1*) Enhancer-Derived Noncoding RNA in the Regulation of *Gnrh1* Gene Expression

by

Polly Pu Huang

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2016

Professor Pamela L. Mellon, Chair

Gonadotropin-releasing hormone (GnRH), a neuropeptide released from a small population of neurons in the hypothalamus, is the central mediator of the hypothalamic-pituitary-gonadal axis, and is required for normal reproductive development and function. Evolutionarily conserved regulatory elements in the mouse, rat, and human *Gnrh1* gene include three enhancers and the proximal promoter, which confer *Gnrh1* gene expression specifically in GnRH neurons. In immortalized mouse hypothalamic GnRH (GT1-7) neurons, which show pulsatile release of GnRH in culture, RNA sequencing and RT-qPCR revealed that expression of a novel long noncoding RNA at *Gnrh1* enhancer 1 correlates with high levels of *Gnrh1* mRNA expression. In GT1-7 neurons, which contain a transgene carrying 3 kb of the rat *Gnrh1* regulatory region, both the mouse and rat GnRH-E1 RNAs are expressed. I investigated the characteristics and function of the

mouse *Gnrh1* enhancer-derived noncoding RNA (GnRH-E1 RNA). Strand-specific RT-PCR analysis of GnRH-E1 RNA in GT1-7 cells revealed sense and antisense GnRH-E1 RNAs that are transcribed from distinct 5' start sites, are 3' polyadenylated, and are over 2 kb in length. GnRH-E1 RNAs are long intergenic noncoding RNAs localized in the nucleus with a half-life of 8 hours. In GT1-7 neurons, siRNA knockdown of mouse GnRH-E1 RNA resulted in a significant decrease in the expression of the *Gnrh1* primary transcript and *Gnrh1* mRNA. Over-expression of either the mouse or rat GnRH-E1 RNA in GT1-7 cells caused a decrease in the transcriptional activity of co-transfected rat *Gnrh1* regulatory elements, which suggests a critical role of balanced GnRH-E1 RNA abundance. The action of GnRH-E1 RNA appears to require critical locations in the *Gnrh1* regulatory region. However, the over-expression of either the sense or antisense variants of the mouse GnRH-E1 RNA in immature, migratory GnRH (GN11) neurons, which do not express GnRH-E1 RNA or *Gnrh1* mRNA, induced the transcriptional activity of co-transfected rat *Gnrh1* gene regulatory elements, where the induction requires the presence of the rat *Gnrh1* promoter. Together, these data indicate that GnRH-E1 RNA functions as an inducer of *Gnrh1* gene transcriptional activity. GnRH-E1 RNA may play an important role in the development and maturation of GnRH neurons.

INTRODUCTION

The reproductive neuroendocrine axis

Reproductive development and fertility are controlled by the dynamic mechanisms of the reproductive neuroendocrine axis. In mammals, the reproductive neuroendocrine axis consists of the hypothalamus in the brain, the anterior pituitary, and the gonads, and is also known as the hypothalamus-pituitary-gonadal (HPG) axis. In the adult, gonadotropin-releasing hormone (GnRH) decapeptide is secreted in pulses from a small population of neurons in the hypothalamus (1). GnRH neurons extend axon terminals to the median eminence, where GnRH is released into the portal blood. GnRH binds to receptors on gonadotropes in the anterior pituitary and stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH stimulate the gonads for growth, maturation, and steroid hormone secretion. Steroid hormone feedback can either inhibit or stimulate GnRH release, FSH and LH depending on gender and timing. Together, the brain, the anterior pituitary, and the gonads form a feedback loop that dynamically controls balanced endocrine function.

GnRH is at the top of the neuroendocrine axis, as the master regulator of reproductive function. GnRH neurons integrate a variety of signals from other neural pathways that represent physiological states, including stress, circadian rhythms, metabolic and energy status, and sex steroid hormone levels. Stress signals have been shown to repress reproductive capacity through suppression of hypothalamic GnRH secretion (2, 3) and GnRH sensitivity of the pituitary gonadotropes (4). Circadian rhythms regulate hypothalamic GnRH secretion (5, 6) and *Gnrh1* gene expression (7, 8), which contributes to seasonal reproductive cycles in mammals and changes in reproductive capacity due to circadian disruptions (9). A complex network of peripheral

sensory pathways and central neural pathways integrate metabolic and energy balance in the modulation of the HPG axis (10). During development and in adulthood, coordinated steroid hormone feedback to the brain and the pituitary dynamically controls the activity of the HPG axis. During development, changes in *Gnrh1* expression are correlated with the timing of pubertal development (11, 12). The increase in amplitude and frequency of GnRH release at puberty is a definitive hallmark of reproductive maturation (13, 14). In the adult female estrous cycle, for instance, fluctuations of estrogen and progesterone dynamically control GnRH release from the hypothalamus, resulting in the LH surge which is critical for ovulation (15). Kisspeptin (Kiss1) release from neurons in the hypothalamus and Kiss1 activation of its receptor (Kiss1R) on GnRH neurons are critical for the reproductive development and function (16, 17). Neurokinin B (NKB) and dynorphin A are co-expressed in kisspeptin neurons in the arcuate nucleus of the hypothalamus, and have also been implicated in the control of pulsatile GnRH secretion (18-20).

During embryonic development, GnRH neurons are born in the olfactory placode and migrate to the developing forebrain, as GnRH neurons reach maturation (21). In adulthood, GnRH neurons are found scattered throughout the hypothalamus, forming a population of approximately 800 neurons that secrete GnRH decapeptide in a pulsatile fashion. In the mouse, rat, and human the expression of gonadotropin-releasing hormone 1 (*Gnrh1*) gene is highly specific to hypothalamic GnRH neurons. The dynamic control of *Gnrh1* gene expression during GnRH neuron maturation, migration, and in normal physiological states, has a major impact on reproductive development and function.

Reproductive deficiencies and infertility can be attributed to mutations in genes involved in GnRH neuron migration and maturation, *Gnrh1* gene expression, and GnRH neuron function. Idiopathic hypogonadotropic hypogonadism (IHH) is a multigenic disorder resulting from abnormal GnRH secretion or insensitivity to GnRH. IHH is characterized by delayed or absent sexual development, low gonadotropin and low sex steroid hormones. Kallmann Syndrome is an X-linked disorder caused by mutation in the *Kal1* gene, and is a known cause of IHH. Kallman Syndrome is characterized by failed GnRH neuron migration and maturation, as well as anosmia and infertility (22). Other causes of IHH patients include mutations in the GnRH receptor gene (*Gnrh1R*), mutations in neuropeptide kisspeptin (*Kiss1*) gene and its receptor gene (*Kiss1R*), and mutations in the neurokinin B gene *TAC3* and its receptor gene *TACR3*. These indicate that IHH result from the failure of GnRH neuron stimulation by kisspeptin or neurokinin B neurons (23). Failure of GnRH neuron maturation is also implicated in Prader-Willi Syndrome (24) and CHARGE Syndrome (25), along with additional phenotypes. However, these reproductive deficient states result from multi-genic causes, and phenotypes often overlap. The majority of genetic causes and molecular mechanisms involved in the pathology of IHH-related reproductive deficiencies remain unclear. Thus, it is critical to elucidate the mechanisms of *Gnrh1* gene regulation to uncover the causes of IHH-related infertility.

The research described in this Dissertation explores a novel molecular mechanism of *Gnrh*1 gene regulation. Understanding the mechanisms regulating GnRH neuron maturation and migration, as well as *Gnrh1* gene expression and GnRH secretion will provide important insight into the physiology and pathophysiology of mammalian reproduction.

Cell models for the study of Gnrh1 *gene regulation*

The size and distribution of the unique population of GnRH neurons in the hypothalamus presents a challenge to the study of GnRH neuron function and *Gnrh1* gene regulation. Cell models of GnRH neurons serve as important tools for investigating the molecular mechanisms that control *Gnrh1* gene expression. Immortalized, mature, mouse hypothalamic GnRH (GT1-7) neurons were generated using a transgene containing 3 kb of rat *Gnrh1* 5' regulatory region driving an simian virus 40 (SV40) T-antigen oncogene in mice (26) to produce tumors in the hypothalamus. GT1-7 neurons represent differentiated GnRH neurons that express high levels of GnRH mRNA and secrete GnRH in a pulsatile manner. Immature, migratory GnRH (GN11) neurons were generated in mice using a transgene containing 1.1 kb of the human *GNRH1* 5' regulatory region on SV40 T-antigen (27) to produce a tumor in the nose. GN11 cells do not express appreciable *Gnrh1* mRNA and do not secrete GnRH (28), but do respond to migratory cues in culture. These cell lines have been validated in numerous models and studies of the role of GnRH neurons in reproductive function.

Regulation of the Gnrh1 *gene*

Evolutionarily conserved regulatory elements of the mouse, rat, and human genes that encode GnRH include three enhancers and the proximal promoter. Together, the *Gnrh1* regulatory elements confer specific expression of *Gnrh1* in the discrete population of hypothalamic GnRH neurons (29). As shown in the rat gene by transfection into mouse GT1-7 cell lines, GnRH-E1 interacts with GnRH-E2, GnRH-E3, and GnRH-P

in specifying GnRH neuron-specific gene expression, and lack activity in GN11 and NIH3T3 mouse fibroblast cells (30, 31).

Transient transfection of plasmid reporters carrying the rat GnRH-E3, E2, and E1 shows significantly higher reporter activity in GT1-7 neurons, compared to GN11 and NIH3T3 cells. Furthermore, transient transfection of plasmid reporter carrying only E3 and E2 in GT1-7 cells shows significantly lower reporter activity than a transfected plasmid reporter containing all three enhancer elements. Thus, GnRH-E1 plays a critical role in concert with the *Gnrh1* promoter to drive robust *Gnrh1* gene expression specifically in mature GnRH neurons and contribute to the absence of *Gnrh1* gene expression in immature, migratory GnRH neurons (30, 32).

The essential role of GnRH-E1 in mature GnRH neuron-specific *Gnrh1* transcriptional activity is also observed *in vivo*. Transgenic mice carrying rat GnRH-E1 and GnRH-P driving β-galactosidase showed GnRH neuron-specific expression, while GnRH-E1 on a heterologous promoter showed non-hypothalamic reporter gene expression in the brain (33). Knock-out mice carrying a deletion of the endogenous GnRH-E1 showed decreased *Gnrh1* mRNA expression in the brain, but showed higher ovarian *Gnrh1* mRNA expression accompanied by irregular estrous cyclicity (34). These reports further highlight the functional significance of *Gnrh1* enhancer 1 and the relationship with *Gnrh1* promoter in the specificity of GnRH gene expression.

A number of transcription factors and homeodomain proteins have been shown to bind at the rat *Gnrh1* enhancer region. OCT1, a POU homeodomain protein, and TALE homeodomains PREP1/PBX binding at the *Gnrh1* regulatory region is observed in GT1-7 cells, but not GN11 and NIH3T3 cells, suggesting neuron-specific control of *Gnrh1* expression (35-37). The zinc finger protein GATA-4 was also shown to bind to the

rat *Gnrh1* regulatory region (-1571 to -1863) (38, 39) during GnRH neuron migration in development, but is not found in adulthood (40), which suggests temporal regulation of *Gnrh1* expression. In addition, Q50 non-Hox homeodomain transcription factors MSX and DLX bind directly to CAATTA repeat elements in both GnRH-E1 and promoter. In GT1-7 cells, co-transfection of MSX1 or 2 and reporter plasmid carrying *Gnrh1* enhancer and promoter showed repression of *Gnrh1* promoter activity, while DLX2 or 5 relieved the repression, suggesting dynamic control of enhancer and promoter activity. In fact, mice lacking MSX or DLX showed abnormal numbers and spatial distribution of GnRH neurons throughout development, suggesting critical role of these transcription factors at the *Gnrh1* regulatory region (41, 42). Two other transcription factors occupy the ATTA repeat elements in both GnRH-E1 and the promoter: homeodomain transcription factors sine oculis-related homeobox (SIX6) and ventral anterior homeobox 1 (VAX1), both of which act as inducers of *Gnrh1* expression and are highly expressed specifically in GT1-7 neurons. Mice lacking SIX6 show dramatically decreased hypothalamic GnRH neuron numbers, disrupted reproductive development, and severely reduced reproductive capacity (43). The absence of VAX1 in GnRH neurons results in infertility and the absence of hypothalamic GnRH neurons observed as early as late embryonic development. Both SIX6 and VAX1 are required for the maintenance of *Gnrh1* expression during GnRH neuron maturation in embryonic development. Interestingly, VAX1 is a relatively modest activator of *Gnrh1* transcription, but competes with SIX6, a strong activator of *Gnrh1* transcription, for occupation at the ATTA repeat element (44).

In the mouse gene, three *Gnrh1* enhancers (GnRH-E1, E2, and E3) and the proximal *Gnrh1* promoter (GnRH-P) reside within 4.8 kb upstream of the mouse *Gnrh1* transcription start site (TSS), as defined by homology to their well-characterized counterparts in the rat *Gnrh1* gene. GnRH-E3 and GnRH-E2 are located within the 3’

untranslated region (UTR) of the *Kctd9* gene. In the mouse gene, GnRH-E3 at -4688 bp/-4385 bp, GnRH-E2 at -3622 bp/-3100 bp, and GnRH-E1 at -2404 bp/-2100 bp facilitate the transcriptional activity at the *Gnrh1* promoter (GnRH-P) at -278 bp/-97 bp. Chromatin modifications at the *Gnrh1* regulatory elements dynamically control *Gnrh1* gene expression (45).

Transcription at enhancers

Enhancers are defined as distal regulatory DNA elements, characterized by DNA-binding protein occupancy and positive regulation of proximal genes. The physical interaction between enhancers and proximal promoters promote tissue-specific gene expression. Enhancers also play an important role in the recruitment of transcription co-activators and transcription factors to interact with the proximal promoter (46-48). Transcription at enhancers has been described to be a genome-wide phenomenon in a constitutively active and regulated manner (49, 50). Actively transcribed enhancers display signature histone marks of high H3K4me1 and low H3K4me3 (51), open chromatin structure, transcription factor binding, and RNA polymerase II enrichment (49, 52-54). Furthermore, transcription at enhancers persistently correlates with the transcriptional activity of proximal genes. Thus, transcription at enhancers has been proposed as an important mechanism in the regulation of proximal genes.

Transcription at enhancers produces polyadenylated and non-polyadenylated noncoding RNAs that have been categorized into different subclasses of noncoding RNAs. The sub-categories of noncoding RNAs are separated by some distinctive physical features, but also share similar physical characteristics that prevent overarching categorization.

Long noncoding RNAs (LncRNA) are a prevalent class of noncoding RNAs and are defined as RNA molecules of over 200 nt in length, 5' capped, 3' polyadenylated, and transcribed by RNA Pol II, but having little protein-coding potential. LncRNAs are predominately spliced and are preferentially transcribed in one direction (55). Transcription of lncRNAs can overlap intergenic regions, intronic regions, or even exonic regions of a proximal protein-coding gene, in either the sense or antisense direction (56). In fact, genome-wide analyses show that about one third to one half of lncRNAs are transcribed from regions that overlap protein-coding genes (57, 58). Transcription at enhancers, marked by high H3K4me1 and RNA Pol II enrichment, can generate noncoding RNAs that can be grouped into the category of lncRNAs.

Enhancer RNAs (eRNAs) are a class of noncoding RNAs transcribed at active enhancers that are associated with monomethylated H3K4 and acetylated H3K27 (59). Most eRNAs are transcribed bi-directionally, with transcription start sites that originate from a central transcription start location (53, 60, 61). The majority of the reported eRNAs are characterized as non-polyadenylated, non-spliced RNA molecules of less than 2 kb in length, though exceptions to these rules have also been observed (62). Nevertheless, the pervasive transcription at enhancers, and the observation that eRNA transcription is associated with the activity of proximal genes (50, 63), helped eRNAs to emerge as an important functional component of gene regulation.

Long intergenic noncoding RNAs (lincRNAs) that are transcribed from noncoding regions between protein-coding genes are a subset of the lncRNAs. Like lncRNAs, lincRNAs are predominantly transcribed in one direction, polyadenylated, and spliced. While some lincRNAs can be transcribed from regions overlapping enhancers, the bidirectional transcription of eRNAs starting from a central region is an important feature

that sets eRNAs apart from the category of lincRNAs. In fact, the subset of uni-directional, polyadenylated eRNAs transcribed from enhancers likely includes a mixture of lincRNAs (52).

Because of the common features shared among noncoding RNAs, lncRNAs have emerged as the prevalent class of noncoding RNAs to include RNA molecules that are over 200 nucleotides in length, 5' capped, predominantly 3' polyadenylated, spliced, and are preferentially transcribed in one direction. As there are exceptions to these rules, such as non-polyadenylated lncRNAs, unspliced lncRNAs, or noncoding RNAs that do not meet the 200 nucleotide arbitrary cutoff, the categorization of lncRNAs is not always clear. Nevertheless, a large number of lncRNAs have been functionally described, validated for biological significance, and have been found to play essential roles in physiology and disease (64).

The functions of enhancer RNA and long noncoding RNA

The correlation between active transcription of eRNA and activity of proximal genes suggests that eRNAs may function as enhancers or facilitators of gene transcription. Actively transcribed enhancers are marked by high H3K4me1 and low H3K4me3, while the proximal promoters with correlated transcriptional activity are marked by low H3K4me1 and high H3K4me3 (51). Several studies on eRNA function have shown that the transcription at enhancers, generating bi-directional eRNAs, can respond to cell signaling cascades that also regulate the transcriptional activity of proximal genes. The depletion or repression of transcription at individual enhancers has resulted in decreased expression of specific neighboring protein-coding genes (65-68). Although bi-directional eRNA transcription is observed at enhancers, in a few cases, the

action of one strand of eRNA can sufficiently affect transcriptional activity of the proximal protein-coding gene (60, 66, 69). These studies highlight the functional significance of enhancer transcripts in the regulation of proximal genes, but raise the question of whether proximal gene expression is dependent on eRNA action or simply the transcriptional activity at enhancers alone.

LncRNAs and lincRNAs participate in a variety of biological pathways, and specific lncRNAs utilize unique strategies to regulate gene transcription. LncRNAs function in *cis* or *trans* (70) to activate or repress gene transcription. *Evf2*, for example, is a noncoding RNA transcribed from the enhancer region of *Dlx-5* and *Dlx-6,* and acts in *trans* to increase the transcriptional activity of another homeodomain protein, *Dlx-2*. *Evf2* recruits DLX-2 as a co-activator to increase the activity of the *Dlx-5/6* enhancer in *cis* (69, 71). The lncRNA *RMST* interacts with transcription factor SOX2 as a co-regulator of distal target genes that are involved in neuronal differentiation (72). LncRNAs *HOTAIR* (73) and *HOTTIP* (74) are well-characterized lncRNAs that interact with chromatin-remodeling complexes and act in *trans* to modulate gene transcription (75, 76), among many other lncRNAs that have been shown to participate in epigenetic regulation (77-79). LncRNAs have been shown to recruit transcription factors to activate nearby genes (72, 80), or sequester transcription factors to repress nearby gene transcriptional activity (81).

A few notable studies have shown the functional significance of enhancer-transcribed lncRNAs in the recruitment of transcription factors to mediate enhancer-promoter interaction. LncRNA involvement in recruiting multi-protein transcriptional complexes Mediator and Cohesin mediates enhancer-promoter interaction, or DNA looping (82-84), and activates gene transcription. Others have also shown that depletion

of lncRNAs that mediate enhancer-promoter interaction effectively alters 3-dimensional DNA interaction and decreases proximal gene transcription (60, 85).

Furthermore, lncRNAs are localized in the nucleus and/or the cytoplasm, and the functional modalities of lncRNAs are different in each location. In addition to gene regulation and epigenetic regulation, nuclear lncRNAs have been shown to act as structural scaffold in nuclear domains (86), and maintain 3-dimensional chromosome structure and activity (75, 87, 88). In the cytoplasm, lncRNAs can act as regulators of post-transcriptional mRNA processing (89, 90), or sequester microRNAs (91).

Analyses of genome-wide transcription using high-throughput sequencing technologies revealed that, although only a small fraction (1.2% - 1.5%) of the mammalian genome consists of protein-coding transcripts, the non-coding regions of the genome are not transcriptionally silent (49, 50, 92, 93). In particular, transcription at enhancers is associated with the regulation of protein-coding genes. Although the functional significance of eRNAs and lncRNAs has been implicated in various biological pathways, the functional mechanisms of specific eRNAs and lncRNAs require extensive biological validation. The research described in this Dissertation provides a functional study of a novel noncoding RNA in the context of *Gnrh1* gene regulation and mammalian reproduction, and will serve as a positive contribution to the wealth of knowledge about noncoding RNA function.

The human lincRNA located upstream of GNRH1

The human lincRNA RP11-395l14.2 (ENSG00000253476) is located in the in the intergenic region between *KCTD9* and *GNRH1*. The human lincRNA RP11-395l14.2 is a

528 bp noncoding RNA with 2 exons and is transcribed in the same direction as *GNRH1*. The human lincRNA RP11-395l14.2 expression profile does not appear to be exclusive to the brain and the function of this human lincRNA is unknown. A portion of RP11-395l14.2 that contains exon 2 and an intronic region shares homology with the -1879 bp/-1340 bp region of the mouse *Gnrh1* gene, but whether the mouse and human lincRNAs share functional similarities is unknown.

The discovery of Gnrh1 *enhancer-derived noncoding RNA*

The initial discovery *Gnrh1* enhancer-transcribed RNA highlighted the characteristics of actively transcribed enhancers. The mouse *Gnrh1* gene regulatory region displays DNase1 hypersensitivity, histone markers of actively transcribed enhancers (i.e. high H3K4me1 and low H3K4me3), and RNA polymerase II (Pol II) enrichment primarily in GT1-7 neurons, whereas histone markers of inactive chromatin were observed in GN11 and NIH3T3 cells. Treatment of GT1-7 cells with 12-O-tetradecanoyl-phorbol-13-acetate (TPA), an activator of the protein kinase C (PKC) pathway, repressed *Gnrh1* mRNA expression and reduced RNA Poll II occupancy at the mouse GnRH-E1. RT-PCR analyses showed the expression of the *Gnrh1* enhancer-derived RNA (GnRH-E1 RNA) robustly correlated with *Gnrh1* mRNA expression in a GT1-7 neuron-specific manner and in response to PKC pathway signaling after TPA treatment (45). These initial observations suggested functional significance of a novel noncoding RNA transcribed from an active enhancer in cell type-specific manner.

As summarized above, the discovery of a novel noncoding RNA in the *Gnrh1* regulatory region identified a potential regulator of *Gnrh1* gene expression, with implications in our understanding of the molecular mechanisms that govern reproductive

development and function. The characterization of GnRH-E1 RNA would expand our knowledge about the function of noncoding RNAs and the role of noncoding RNAs in GnRH neuron function. The research presented herein establishes the foundation for future studies of a novel regulator of *Gnrh1* gene expression in cell models, with applications in our overall understanding of reproductive development and physiology.

CHAPTER 1. The Expression and Function of the *Gnrh1* Enhancer-Derived Noncoding RNA in *Gnrh1* Gene Regulation

1.1 Introduction

Gonadotropin-releasing hormone (GnRH) neurons are at the apex of the reproductive neuroendocrine axis. During development, GnRH neurons originate in the olfactory placode, and migrate to the developing forebrain. In adulthood, mature GnRH neurons are found scattered throughout the hypothalamus and form a small population of neurons that secrete GnRH decapeptide in a pulsatile fashion (1). Proper regulation of gonadotropin-releasing hormone (*Gnrh1*) gene expression and the coordinated action of the hypothalamic-pituitary-gonadal axis govern normal reproductive development and function, including puberty, menstrual cycle, pregnancy, and menopause. Reproductive disorders such as Kallmann Syndrome (22), Idiopathic Hypogonadotropic Hypogonadism (23), Prader-Willi Syndrome (24), and CHARGE Syndrome (25), are attributed to disruptions in *Gnrh1* gene expression, GnRH neuron signaling, and/or GnRH neuronal maturation. However, the majority of genetic causes and molecular mechanisms involved in the pathology of these reproductive deficiencies remain undefined. Thus, understanding the mechanisms that modulate *Gnrh1* gene expression will provide important insights into the biology and pathophysiology of mammalian reproduction.

We used GnRH model cell lines in culture to study the molecular mechanisms of *Gnrh1* gene regulation. Immortalized hypothalamic GnRH (GT1-7) neurons were generated using a transgene containing 3 kb of rat *Gnrh1* 5’ regulatory region driving an oncogene (SV40 T-antigen) in mice (26) to produce tumors in the hypothalamus. GT1-7 neurons represent mature, differentiated GnRH neurons that express high levels of *Gnrh1* mRNA and secrete GnRH in a pulsatile manner (7, 94-96). GN11 neurons were

generated in mice using a transgene containing 1.1 kb of the human *Gnrh1* 5' regulatory region on SV40 T-antigen (27) to produce a tumor in the nose. GN11 cells represent immature, migratory GnRH neurons, which do not express *Gnrh1* mRNA and do not secrete GnRH (97).

Conserved regulatory elements in the mouse, rat, and human *Gnrh1* gene include three enhancers and the proximal promoter that, together, confer specific expression of *Gnrh1* in the discrete population of hypothalamic GnRH neurons. Three *Gnrh1* enhancers and the proximal promoter reside within 4.8 kb upstream of the mouse *Gnrh1* gene transcription start site (TSS) as defined by homology to their well-characterized counterparts in the rat gene. In the mouse gene, *Gnrh1* enhancer 3 (GnRH-E3) at -4688 bp/-4385 bp, enhancer 2 (GnRH-E2) at -3622 bp/-3100 bp, and enhancer 1 (GnRH-E1) at -2404 bp/-2100 bp facilitate transcriptional activity of the *Gnrh1* promoter (GnRH-P) at -278 bp/-97 bp (31). Importantly, as shown in the rat gene by transfection into the mouse GT1-7 cell line, GnRH-E1 interacts with GnRH-E2, GnRH-E3, and GnRH-P in specifying GnRH neuron-specific gene expression (29, 98).

GnRH-E1 is critical for specifying the robust *Gnrh1* gene expression in mature GnRH neurons and the absence of *Gnrh1* gene expression in immature, migratory GnRH neurons (30, 32). Transgenic mice carrying the rat GnRH-E1 and GnRH-P driving β-galactosidase showed GnRH neuron-specific expression, while rat GnRH-E1 on a heterologous promoter showed non-specific β-galactosidase expression in the brain (33). Knock-out mice carrying a deletion in the endogenous mouse GnRH-E1 showed decreased *Gnrh1* mRNA expression in the brain, but showed higher ovarian *Gnrh1* mRNA expression accompanied by irregular estrous cyclicity (34). These reports

highlight the functional significance of *Gnrh1* enhancer 1 and the relationship with *Gnrh1* promoter in the specificity of *Gnrh1* gene expression.

Constitutively active and signal-regulated transcription at enhancers has been described to be a genome-wide phenomenon that is associated with the regulation of gene expression. Actively transcribed enhancers display signature histone marks of high H3K4me1 and low H3K4me3 (51), open chromatin structure, and RNA polymerase II (RNA Pol II) enrichment (99, 100). Transcription at enhancers can generate functional noncoding RNAs with different transcriptional modalities and structures. Long noncoding RNA (LncRNA) transcription can overlap intergenic regions, introns or exons of protein-coding genes (56), thus lncRNA transcription can overlap enhancers. LncRNA are 5' capped and 3' polyadenylated RNA molecules of over 200 nt in length. LncRNAs are predominately spliced and are transcribed in one direction. Long intergenic noncoding RNAs (lincRNAs), a subset of lncRNAs, are transcribed from regions between genes, including enhancers. On the other hand, transcription at enhancers can produce enhancer RNAs (eRNAs), which are categorized as a sub-class of lncRNAs. Transcription of eRNAs is generally bi-directional from a central region, producing relatively short (0.5 kb – 2 kb) noncoding transcripts that predominantly lack polyadenylation or splicing (50). While RNA transcription at enhancers has been observed as part of the genome-wide transcriptional landscape, the functional significance of the majority of individual noncoding RNAs and their effect on individual gene regulation remains to be elucidated.

The initial discovery of *Gnrh1* enhancer-transcribed RNA highlighted the characteristics of actively transcribed enhancers. The endogenous *Gnrh1* regulatory region displays DNase1 hypersensitivity, histone markers of actively transcribed

enhancers (i.e. high H3K4me1 and low H3K4me3), and RNA pol II enrichment primarily in GT1-7 neurons, whereas histone markers of inactive chromatin were observed in GN11 and NIH3T3 cells as assayed by chromatin immunoprecipitation (ChIP). Treatment of GT1-7 cells with 12-O-tetradecanoyl-phorbol-13-acetate (TPA), an activator of the protein kinase C (PKC) pathway, repressed *Gnrh1* mRNA expression and reduced RNA Poll II occupancy at GnRH-E1. RT-PCR analyses showed the expression of *Gnrh1* enhancer-transcribed RNA robustly correlated with *Gnrh1* mRNA expression in a GT1-7 neuron-specific manner and in response to PKC pathway signaling after TPA treatment (45). These initial observations suggest functional significance of a novel noncoding RNA transcribed from an active enhancer in cell type-specific manner. In this study, we characterized the expression profile and function of the *Gnrh1* enhancer-derived noncoding RNA (GnRH-E1 RNA) in GnRH model cell lines. We establish GnRH-E1 RNA as a novel regulator of *Gnrh1* gene expression. Our data provide the foundation for future studies on the molecular mechanisms of *Gnrh1* regulation with implications in mammalian reproductive development and fertility.

1.2 Materials and Methods

Cell culture, actinomycin D treatment, and cell fractionation

GT1-7, GN11 (courtesy of Dr. Sally Radovick, Rutgers University, and Susan Wray, National Institutes of Health), and NIH3T3 cell lines were cultured in DMEM containing 4.5% glucose (Mediatech Inc., Herndon, VA), 10% fetal bovine serum (Gemini Bio Products, West Sacramento, CA), and 1% penicillin-streptomycin cocktail (Hyclone Laboratories, Logan, UT) in 5% CO_2 at 37°C. Cell lines at passage 15-25 were used in this study. For actinomycin D treatments, GT1-7 cells were seeded in 10 cm

plates (Nunc, Roskilde, Denmark) at 300,000 cells/mL 24 hours prior to treatment. Cells were treated with 1 µg/mL actinomycin D or 0.01% DMSO (Sigma Aldrich, St. Louis, MO) vehicle. At the time points indicated, cells were rinsed with phosphate-buffered saline (PBS) and homogenized in TRIzol® Reagent (Thermo Fisher Scientific, Carlsbad, CA) for RNA extraction.

To harvest nuclear and cytoplasmic extracts from GT1-7 neurons in culture, cells were first aspirated and washed with PBS, followed by centrifugation at 1000 x *g* for 5 minutes. The cell pellet was resuspended in cytoplasmic extract buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% NP40, 1 mM DTT, and 1 mM PMSF) on ice for 3 minutes. After centrifugation at 1500 x *g* for 4 minutes, the cytoplasmic extract (supernatant) was homogenized in TRIzol Reagent for RNA extraction. The pellet containing nuclei was resuspended in cytoplasmic extract buffer without NP40. After centrifugation of the nuclei at 1500 x *g* for 4 minutes, the buffer was removed and the nuclei were resuspended in nuclear extract buffer (20 mM Tris at pH 7.9, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 1 mM PMSF) and incubated on ice for 10 minutes. After centrifugation at 13,000 *x g* for 10 minutes, the nuclear extract buffer was removed and the pellet containing nuclei was homogenized in TRIzol Reagent for RNA extraction.

Reverse transcription-PCR and quantitative PCR

Total RNA was harvested from GT1-7, GN11, and NIH3T3 cells using TRIzol Reagent according to the manufacturer's recommendations. Genomic DNA was removed from RNA samples using TURBO DNA-free™ DNase Kit (Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturer's recommendations. First-strand cDNA was synthesized from the total RNA from each cell line, using SuperScript® III First-Strand cDNA Synthesis System (Thermo Fisher Scientific, Carlsbad, CA),

according to the manufacturer's recommendations, and was primed by either oligo$(dT)_{20}$ primers (from cDNA synthesis kit) or gene-specific primers (IDT DNA Technologies, San Diego, CA), where indicated. Gene-specific primers for cDNA synthesis are listed in Table 1.

PCR was performed using FastStart™ *Taq* DNA Polymerase (Roche Diagnostics, Indianapolis, IN) and the following conditions: 95°C for 5 minutes denature, followed by 30 cycles of 95°C for 45 seconds, 60°C for 45 seconds, 72°C for 45 seconds, and final extension of 72°C for 10 minutes. PCR primers are listed in Table 1 and were tested for optimal annealing temperatures and PCR conditions. RT-PCR products were labeled by the addition of 10 µg/mL of ethidium bromide and were resolved on a 1% agarose gel for >1 kb PCR product and 2% agarose gel for ≤ 500 bp PCR product in 1X TAE buffer (40 mM Tris at pH 7.6, 20 mM acetic acid, and 1 mM EDTA).

Quantitative PCR was performed using iQ™ SYBR® Green Supermix and iQ5 Real-Time PCR detection system and software (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. Standard curves were generated using serial dilutions of plasmid DNA containing the PCR amplicon cloned into pcDNA2.1 backbone. Quantities of GnRH-E1 RNA, *Gnrh1* pre-mRNA, and *Gnrh1* mRNA were normalized to histone 2A.Z (*H2afz*) mRNA control or peptidylprolyl isomerase A (*Ppia*) mRNA control, where indicated. RT-qPCR primers are listed in Table 1.

RNA-sequencing

RNA was isolated from GT1-7, GN11, and NIH3T3 cells using TRIzol® Reagent according to the manufacturer's instructions, and treated with TURBO DNA-free™ DNase. Concentration was determined by qRT-PCR. The RNA integrity (RNA Integrity

Table 1. Oligonucleotide sequences.

Primer Name	*	Sequence (5' to 3')
Mouse GnRH-E1 RNA -1700	F	GCGTCAAACTACAAAGGAAAGTGGCTGCAT
Mouse GnRH-E1 RNA -3371	F	GGGAAAGGAAAGCAATTTCA
Mouse GnRH-E1 RNA -3560	F	AGCTGTGTCCAAATGGGTTC
Mouse GnRH-E1 RNA -3606	F	CGTAGGTGTCCCAGTGTCCTTATTTGTTGC
Mouse GnRH-E1 RNA -3746	F	CTCTATGTCAGTACCTGTGGCTCGGGCTCG
Mouse GnRH-E1 RNA -2438	F	GCGTCAAACTACAAAGGAAAGTGGCTGCAT
Mouse GnRH-E1 RNA -2301	F	GTTGTTCACCCATCATTCAGGAAGGTTACA
Mouse GnRH-E1 RNA -2068	F	GCCTCAAAACCTCACTGGCTGCTTAATGG
Mouse GnRH-E1 RNA -2106	R	TAGGCCAATTTACCCAAGACCTGAAATTGA
Mouse GnRH-E1 RNA -1271	R	GCAGATGCTGCCTCTATTTCA
Mouse GnRH-E1 RNA -1128	R	GCCTGCCTCTGAAACTTTTG
Mouse GnRH-E1 RNA -1443	R	TGCTAACCCCAATCCGCCATTGCTT
Rat GnRH-E1 RNA -1640	F	GCAGACAGAGGAGCTGAGAGATGCAAAC
Rat GnRH-E1 RNA -1385	R	AGGGTAGGGAAGAGGGACTTTGTGGTG
Rat GnRH-E1 RNA -2854	F	GCAGACAGAGGAGCTGAGAGATGCAAAC
Rat GnRH-E1 RNA -1156	R	AGGGTAGGGAAGAGGGACTTTGTGGTG
Gnrh1 mRNA +1144 (exon)	F	CTACTGCTGACTGTGTGTTTG
Gnrh1 mRNA +4059 (exon)	R	CATCTTCTTCTGCCTGGCTTC
Gnrh1 pre-mRNA +1297 (intron)	R	GCTCATGCAGTTTGAAGCGAGTC
H2afz mRNA	F R	TCACCGCAGAGGTACTTGAG GATGTGTGGGATGACACCA
Ppia mRNA Forward	F R	AAGTTCCAAAGACAGCAGAAAAC CTCAAATTTCTCTCCGTAGATGG
siRNA sense		AAACUACAAAGGAAAGUGG
siRNA antisense		CCACUUUCCUUUGUAGUUU
siRNA negative control pool		UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUUCCUA

* F indicates forward direction and R indicates reverse direction.

Number ≥ 9) and quantity was determined on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). cDNA libraries were created using the TruSeq™ RNA Sample Prep-v2 (Illumina, San Diego, CA), using the manufacturer's low-throughput protocol. Indexed samples were mixed at equal concentrations, four samples per lane, and sequenced using the HiSeq 2000 sequencer (Illumina, San Diego, CA). The resulting Fastq sequence reads were validated and analyzed in Galaxy (101) using FASTQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and FASTQ Groomer (102), and any remaining adapter sequences removed using FASTQ Clipper program (http://hannonlab.cshl.edu/fastx_toolkit/index.html). The reads were aligned to the mouse genome (mm10 assembly) using the TopHat2 program (103) and differential expression analyzed using Cufflinks software program (104). Sequence read alignments (accepted-hit bam files) were visualized using the Integrative Genome Viewer (105, 106). Sequencing library preps, qRT-PCR, sequencing, and alignment were performed by the UCSD BIOGEM Core facility supported by NIH grants P30 DK063491 and P30 CA023100.

Northern blot

Total RNA from GT1-7, GN11, and NIH3T3 cells were extracted using TRIzol Reagent, and 30 µg of RNA was prepared for Northern blotting in reaction mixture (0.1 M MOPS, 40 mM sodium acetate, 5 mM EDTA, formaldehyde, formamide, and 1 mg/mL ethidium bromide). RNA samples were incubated at 65°C for 15 minutes before being loaded into 1% agarose formaldehyde gel (4% formaldehyde in 0.1 M MOPS, 40 mM sodium acetate and 5 mM EDTA). Resolved RNA on the gel was transferred to a nylon membrane (Thermo Fisher Scientific, Carlsbad, CA) in 20X sodium sulfate citrate buffer. The transfer apparatus was constructed as described in Molecular Cloning: A Laboratory

Manual (107), and the transfer was allowed to proceed overnight. RNA was cross-linked onto the blot using UV light in the Stratalinker (Stratagene, La Jolla, CA), according to the manufacturer's instructions. To prepare the DNA probe targeting GnRH mRNA, the previously described (108) pBluescript II SK (-) plasmid (Stratagene, La Jolla, CA) containing the PCR clone of 370 bp of the mouse *Gnrh1* (+120 bp/+489 bp) was linearized before radiolabeling. The plasmid containing the GnRH-E1 RNA probe (described below) was linearized before radiolabeling. DNA probes targeting GnRH-E1 RNA and GnRH mRNA were radiolabeled with [α-^{32}P] dATP (Perkin Elmer, Waltham, MA) using DECAPrime™ DNA labeling kit (Thermo Fisher Scientific, Carlsbad, CA), according to the manufacturer's recommendations. Pre-hybridization, hybridization, and washing of the Northern blot was performed as previously described (109). Northern blot developed by autoradiography on X-ray film for 24 hours at -70°C.

Plasmids

The -5 kb *Gnrh1*-luciferase reporter and 5' truncations were generated as previously described (31). The rat *Gnrh1* enhancer 1 and promoter (GnRH-E1/GnRH-P) luciferase reporter in the pGL3 vector has been previously described (42, 110). The luciferase reporters carrying the Rous sarcoma virus enhancer and promoter (RSVe/RSVp), GnRH-E1/RSVp, RSVe/GnRH-P, and GnRH-E1/RSVp in the pGL3 vector have been previously described (30). The Luciferase reporters carrying the rat *Gnrh1* enhancers (E3, E2 and E1), combinations of two enhancers, or one enhancer alone, appended to the heterologous promoter RSVp have been previously described (31). The luciferase plasmid carrying a multimer of the SIX6 consensus sequence upstream of the thymidine kinase promoter (TKp) has previously been described (43).

The luciferase reporter plasmids carrying multimers of OTX2 consensus sequence found at -152 bp or at -198 bp of the rat *Gnrh1* TSS, appended to TKp, have been previously described (111). Luciferase plasmids carrying GnRH-E2 and GnRH-E1, with mutated OCT1 consensus sequences in either or both enhancers were previously described (112). The luciferase reporter plasmid carrying a multimer of OCT1 consensus sequence has been previously described (113).

The mouse GnRH-E1 RNA expression plasmid was constructed by PCR amplification of the 2432 bp (-3560 bp/-1128 bp) segment from GT1-7 neuron genomic DNA using Platinum® Pfx DNA polymerase kit (Thermo Fisher Scientific, Carlsbad, CA). The segment was inserted at the *EcoR1* restriction enzyme digest site of the pcDNA 2.1 (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) backbone plasmid using T4 DNA ligase (New England Biolabs, Ipswich, MA). Plasmid constructs carrying -3560 bp/-1128 bp of GnRH-E1 RNA, integrated in the forward or the reverse orientation, were verified by DNA sequencing. The rat GnRH-E1 RNA expression plasmid was constructed by PCR amplification of the 1698 bp (-2854 bp/-1156 bp) segment from GT1-7 neuron genomic DNA and inserted at the *KpnI* restriction enzyme digest site of the pcDNA2.1 backbone plasmid, using similar cloning strategy and verification as performed for the mouse GnRH-E1 RNA expression plasmid. For quantitative PCR of the endogenous mouse GnRH-E1 RNA and the rat GnRH-E1 RNA, the respective expression plasmids were utilized as qPCR standards. To construct the DNA probe that detects the mouse GnRH-E1 RNA in the northern blot analysis, a 150 bp probe (-2301 bp/-2106 bp) from the upstream region of the mouse *Gnrh1* TSS was PCR amplified from genomic DNA of dissected mouse hypothalamic tissue and inserted into a TOPO plasmid (Thermo Fisher Scientific, Carlsbad, CA) at *EcoRI* restriction digest sites. Oligonucleotide sequences for cloning the mouse and rat GnRH-E1 RNA expression plasmids are listed in Table 1.

Transfections and reporter assays

For luciferase assays, GT1-7 and GN11 cells were seeded into 24-well plates 24 hours before transfection at concentrations of 250,000 cells/mL and 70,000 cells/mL, respectively. Plasmids were transfected using PolyJet™ Transfection Reagent (SignaGen Laboratories, Rockville, MD) according to the manufacturer's recommendations. Cells were co-transfected with 150 ng/well of luciferase reporter plasmid, 200 ng/well of expression plasmid, and 100 ng/well of thymidine kinase β-galactosidase reporter plasmid as internal control for transfection efficiency. Cells were transfected in parallel with RSVp-luciferase or pGL3-luciferase reporter plasmid where indicated. At 8 hours after transfection, cells were serum starved (DMEM with 0.1% bovine serum albumin and 1% penicillin/streptomycin cocktail) for 24 hours prior to harvest. Cells were harvested using cold lysis buffer (100 mM potassium phosphate at pH 7.8 and 0.2% Triton X-100). Luciferase and β-galactosidase assays were performed using the Galacto-Light Plus System (Thermo Fisher Scientific, Carlsbad, CA).

For siRNA transfections, GT1-7 cells were seeded on 6-well plates at the concentration of 250,000 cells/mL 48 hours prior to transfection. Growth media was replaced with DMEM with 4.5% glucose and 5% fetal bovine serum 24 hours prior to siRNA transfection. On-Target Plus Non-targeting siRNA pool (negative control siRNA), and custom-designed siRNA duplex oligos targeting GnRH-E1 RNA, Lipofectamine® RNAiMax Reagent (Thermo Fisher Scientific, Carlsbad, CA) were prepared according to the manufacturer's recommendations, in OptiMEM® (Thermo Fisher Scientific, Carlsbad, CA) at a concentration of 10 μM. siRNA was transfected into GT1-7 cells at a concentration of 900 pmol per well.

Data analysis

All transient transfections, RT-PCR and quantitative RT-PCR were independently repeated three times or more. For transient transfections, luciferase activity was normalized relative to β-galactosidase to control for transfection efficiency. Unless otherwise noted, the data were normalized to luciferase:β-galactosidase activity ratio of empty pGL3 plasmid transfected in parallel. The data were compared between cells transfected with empty pcDNA2.1 plasmid and those transfected with pcDNA2.1 plasmid carrying GnRH-E1 RNA. Results are presented as mean ± standard deviation (SD) of the fold induction relative to pGL3. Statistical tests were performed on luciferase activity normalized to β-galactosidase. For RT-qPCR, the mean copy number for GnRH-E1 RNA, *Gnrh1* pre-mRNA and *Gnrh1* mRNA were normalized to *H2afz* mRNA or *Ppia* mRNA copy number as internal control, where indidated. Student's t-test and one-way ANOVA followed by Tukey-Kramer HSD post hoc tests were used as indicated, where $p<0.05$ indicated statistical significance.

1.3 Results

Expression of GnRH-E1 RNA in mouse immortalized hypothalamic GnRH neurons

We first examined GnRH-E1 RNA expression using RNA sequencing analysis of model cell lines. The mouse *Gnrh1* gene is located on mouse chromosome 14 and is flanked by the genes *Kctd9* transcribed in the same direction as *Gnrh1* and located upstream of *Gnrh1*, and *Dock5* transcribed in the opposite direction and located downstream of *Gnrh1*. The mouse *Gnrh1* enhancers and promoter are located in the genomic region 5' of *Gnrh1*, where *Gnrh1* enhancers 2 and 3 (E2 and E3) are located in the region overlapping the 3' UTR of *Kctd9,* and *Gnrh1* enhancer 1 (E1) and the promoter are located in the intergenic region 3' of *Kctd9.* The robust detection of RNA

reads that align to the exons of the *Gnrh1* gene confirms the high level of *Gnrh1* expression in GT1-7 cells (Fig. 1A). In contrast, GN11 cells show fewer exonic RNA reads, and *Gnrh1* is virtually not expressed in NIH3T3 fibroblasts (note the different log scale for GT1-7, compared to GN11 and NIH3T3). RNA reads were also detected that align to the introns of the *Gnrh1* gene and likely represent the signal from the *Gnrh1* primary transcript, as the sequencing analysis was performed on total RNA. In addition to the reads aligning to *Gnrh1,* GT1-7 cells showed dense and robust RNA reads that align to the intergenic region of *Gnrh1* and *Kctd9* genes (Fig. 1B). The intergenic RNA reads were indistinguishable from background in GN11 and NIH3T3 cells. Together, these observations are consistent with our previous report of the positive correlation between RNA expression in the upstream regulatory region of the *Gnrh1* gene and GT1-7 cell-specific *Gnrh1* mRNA expression using quantitative PCR analysis (45).

The *Gnrh1* gene regulatory region consists of three enhancers and the promoter located upstream of the *Gnrh1* transcription start site (TSS) (Fig. 2A). GT1-7 neurons also carry a transgene that consists of 3.0 kb of the rat *Gnrh1* regulatory region (Fig. 2B). Due to the high degree of evolutionary conservation between the rat and mouse *Gnrh1* regulatory region (31), we asked whether GnRH-E1 RNA is transcribed from both the mouse endogenous gene and the rat transgene in GT1-7 neurons. RT-PCR analysis of oligo-dT-primed cDNA from GnRH cell models revealed that RNA expression extending upstream and downstream of *Gnrh1* enhancer 1 (GnRH-E1), was present only in GT1-7 neurons and not in GN11 or NIH3T3 cells. Similarly, RNA expression from the rat transgene was correlated with *Gnrh1* mRNA expression only in GT1-7 neurons (Fig. 2C). The data indicate that the rat and mouse GnRH-E1 RNAs are polyadenylated. Additional RT-PCR analysis of GnRH-E1 RNA expression in GT1-7 neurons revealed that the GnRH-E1 RNA transcript extends upstream and downstream of GnRH-E1 from

Figure 1. RNA sequencing analysis of the upstream regulatory region of *Gnrh1*.

A-B) RNA expression in GnRH model cell lines GN11 and GT1-7, and NIH3T3 mouse fibroblasts are displayed in tracks as labeled. The tracks show the number of sequence reads on the y-axis, aligned to chromosome location on the x-axis. The RNA reads are displayed in log scale: 1 - 10^5 for GT1-7 cells, and 1 – 10^2 for GN11 and NIH3T3 cells. A) RNA sequencing analysis of the mouse *Gnrh1* gene and the upstream intergenic region between *Kctd9* and *Gnrh1* is shown. RNA reads are aligned to *Gnrh1*, the upstream intergenic region, and the 3' untranslated region (UTR) of *Kctd9* (mouse Chr14:67,739,815-67,749,849 of the mouse mm10 genome assembly). A schematic diagram of the *Gnrh1* enhancers (E1, E2, E3) and promoter (P) is shown in the GT1-7 track. *Gnrh1* regulatory elements are approximately aligned to the 3' UTR of *Kctd9* and the genomic region that is 5' of *Gnrh1.* B) An enlarged display of RNA reads aligned to the intergenic region located upstream of *Gnrh1* and the 3' UTR of *Kctd9* (mouse Chr14: 67,742,182-67,745,385).

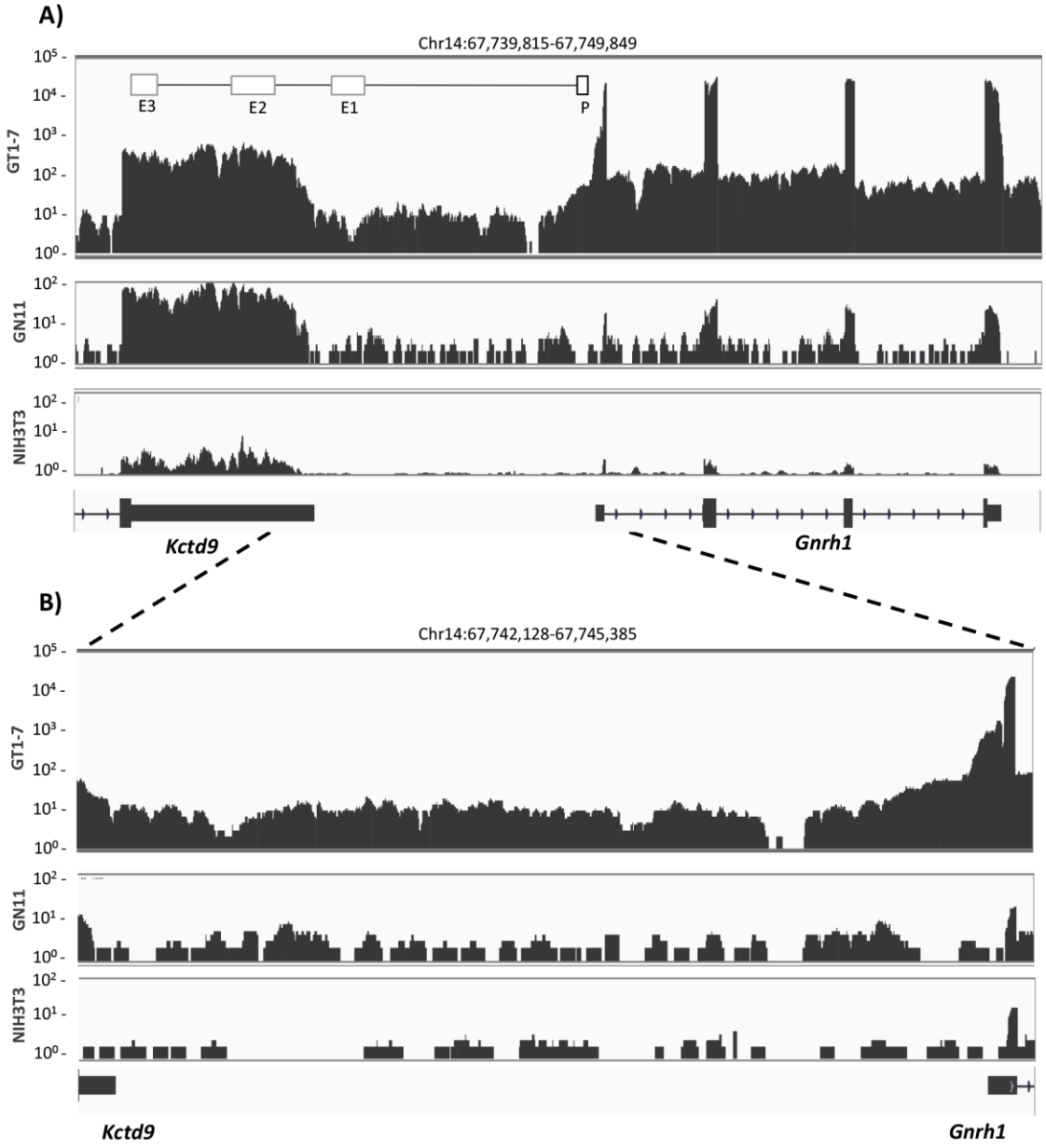
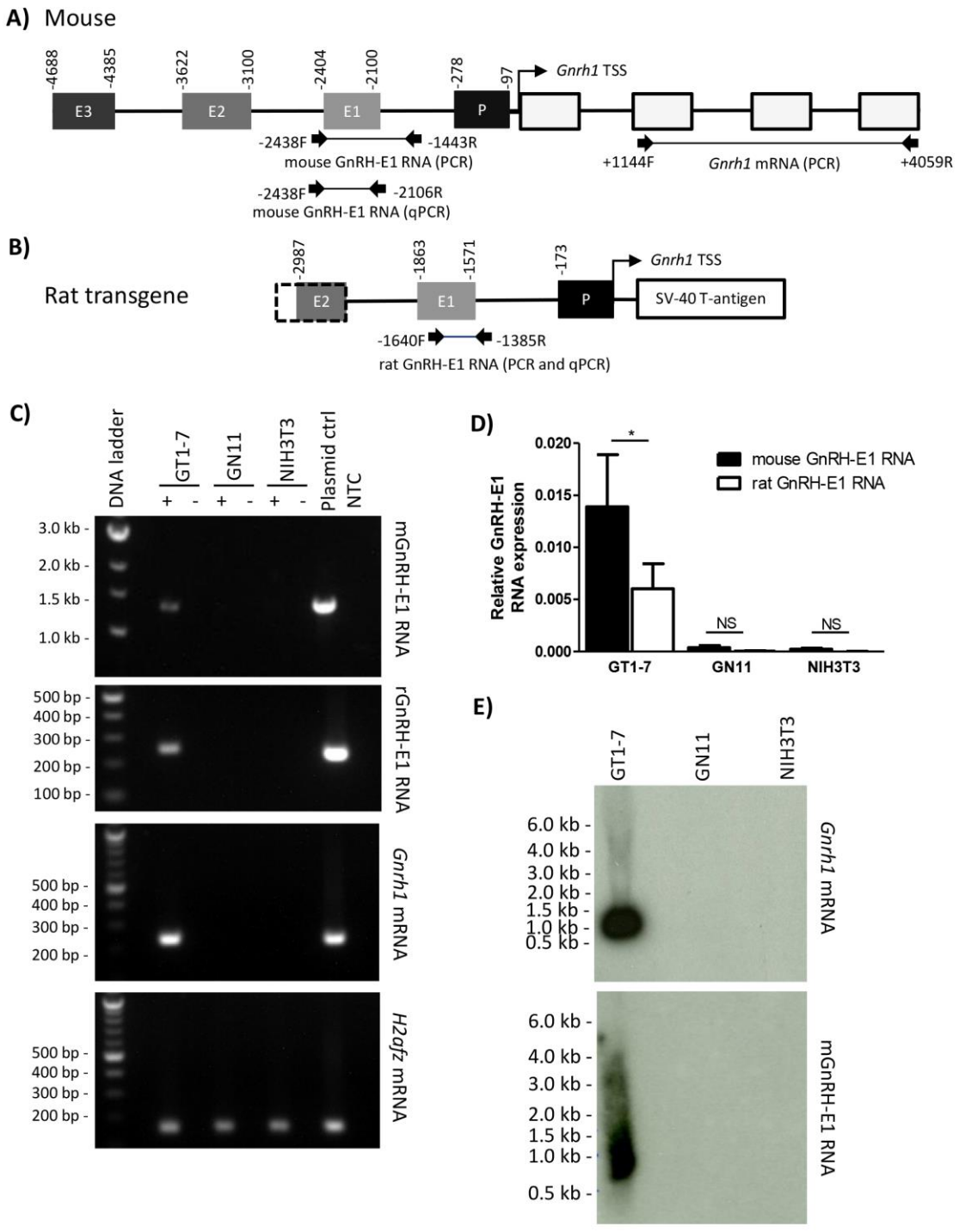

A)
Chr14:67,739,815-67,749,849
E3
E2
E1
P
GT1-7
GN11
NIH3T3
Kctd9
Gnrh1
B)
Chr14:67,742,128-67,745,385
GT1-7
GN11
NIH3T3
Kctd9
Gnrh1

Figure 2. Rat and mouse GnRH-E1 RNA expression in cell lines.

A) Schematic diagram of mouse conserved regulatory elements upstream of the *Gnrh1* transcription start site (TSS, curved arrow) and *Gnrh1* gene coding region. *Gnrh1* gene regulatory region contains *Gnrh1* enhancers 1, 2, and 3 (E1, E2, E3, respectively) and the promoter (P). The *Gnrh1* gene consists of four exons (white boxes). Coordinates above regulatory elements represent positions with respect to the *Gnrh1* TSS. PCR primers used in Fig. 3C-D are indicated by arrows, where the predicted PCR product, mouse GnRH-E1 RNA, is represented by a connecting line. B) Schematic diagram of the rat transgene embedded in GT1-7 neurons carrying the 3' portion of GnRH-E2, GnRH-E1, GnRH-P, and the *Gnrh1* TSS, driving the SV40 T-antigen oncogene. PCR primers used in Fig. 3D are indicated by arrows, where the predicted PCR product, rat GnRH-E1 RNA transcribed from the rat transgene, is represented by a connecting line. Primer sequences are provided in Table 1. C) RT-PCR analysis of mouse GnRH-E1 RNA (mGnRH-E1 RNA), rat GnRH-E1 RNA (rGnRH-E1 RNA), and *Gnrh1* mRNA, and Histone 2A.Z (*H2afz)* mRNA control in GT1-7, GN11 and NIH3T3 cells. Oligo-dT-primed cDNA were synthesized with (+) or without (-) reverse transcriptase, were PCR amplified in parallel with positive, plasmid controls and with negative, no-template control (NTC). D) RT-qPCR analysis of endogenous mouse GnRH-E1 RNA (black) and GnRH-E1 RNA expressed from rat transgene (white) in GT1-7, GN11, and NIH3T3 cells. Relative GnRH-E1 RNA expression is normalized to peptidylprolyl isomerase A (*Ppia*) mRNA control. The data are displayed as means ± SD. Asterisk indicates statistical significance by Student's T-test on the comparison between mouse and rat GnRH-E1 RNA, where $p<0.05$. E) Northern blot analysis of total RNA from GT1-7, GN11 and NIH3T3 cells using DNA probes targeting *Gnrh1* mRNA and endogenous mouse GnRH-E1 RNA.


A) Mouse
-4688
-4385
-3622
-3100
-2404
-2100
-278
-97
Gnrh1 TSS
E3
E2
E1
P
-2438F
-1443R
mouse GnRH-E1 RNA (PCR)
+1144F
Gnrh1 mRNA (PCR)
+4059R
-2438F
-2106R
mouse GnRH-E1 RNA (qPCR)
B)
Rat transgene
-2987
-1863
-1571
-173
Gnrh1 TSS
E2
E1
P
SV-40 T-antigen
-1640F
-1385R
rat GnRH-E1 RNA (PCR and qPCR)
C)
DNA ladder
GT1-7
GN11
NIH3T3
+
-
Plasmid ctrl
NTC
3.0 kb
2.0 kb
1.5 kb
1.0 kb
mGnRH-E1 RNA
500 bp
400 bp
300 bp
200 bp
100 bp
rGnRH-E1 RNA
Gnrh1 mRNA
H2afz mRNA
D)
Relative GnRH-E1 RNA expression
0.020
0.015
0.010
0.005
0.000
*
NS
mouse GnRH-E1 RNA
rat GnRH-E1 RNA
GT1-7
GN11
NIH3T3
E)
6.0 kb
4.0 kb
3.0 kb
2.0 kb
1.5 kb
1.0 kb
0.5 kb
Gnrh1 mRNA
mGnRH-E1 RNA

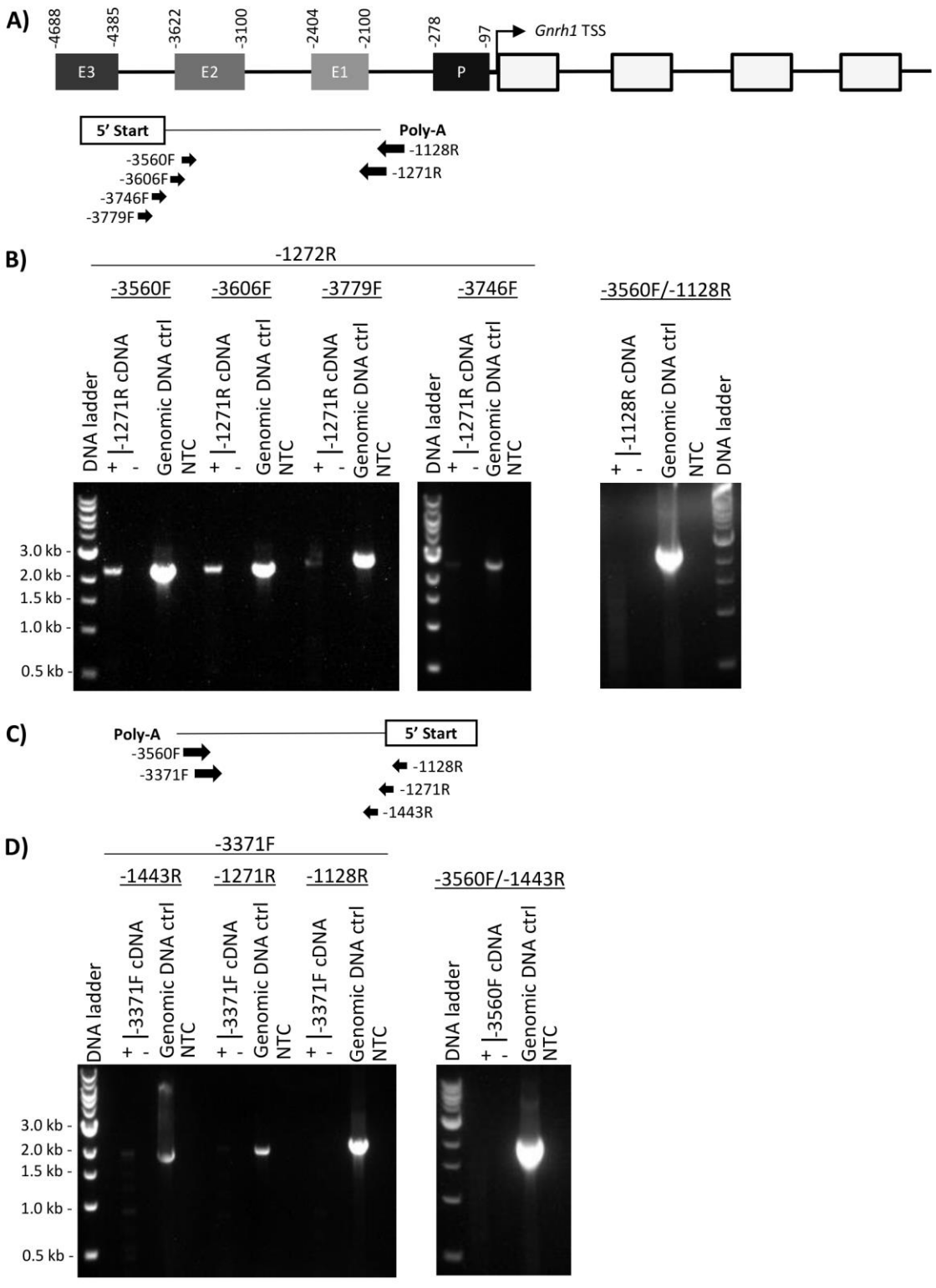
both the endogenous mouse gene and the transgene (data not shown). RT-qPCR analysis further revealed that endogenous RNA expression from the mouse *Gnrh1* regulatory region is significantly higher than RNA expression from the rat transgene in GT1-7 neurons (Fig. 2D).

Northern blot analysis of total RNA from GT1-7, GN11, NIH3T3 cells confirmed GT1-7 neuron-specific expression of *Gnrh1* mRNA and the endogenous mouse GnRH-E1 RNA (Fig. 2E). Neither GnRH-E1 RNA nor *Gnrh1* mRNA was expressed in GN11 or NIH3T3 cells. However, northern blot analysis using a DNA probe targeting both strands of the endogenous mouse GnRH-E1 RNA revealed RNAs of sizes ranging from approximately 700 bases to 3000 bases. Additional RT-PCR analyses, as well as 5' and 3' rapid amplification of cDNA ends (RACE) indicated endogenous mouse GnRH-E1 RNA transcripts extending upstream and downstream of GnRH-E1, with alternate 5' start and 3' termination sites.

We used strand-specific RT-PCR analysis of total RNA from GT1-7 neurons to confirm the length and strandedness of the endogenous mouse GnRH-E1 RNA. To capture the mouse sense GnRH-E1 RNA variant, we used reverse primers at -1128 bp or -1271 bp for first-strand cDNA synthesis. The cDNA synthesized using the gene-specific primer (GSP) at -1271 bp (reverse) was subjected to RT-PCR analysis using the same reverse primer, paired with forward primers at -3560 bp, -3606 bp, -3746 bp, and -3779 bp (Fig. 3A). PCR products of -3560 bp/-1271 bp and -3606 bp/-1271 bp were successfully detected, but PCR products from -3746 bp/-1271 bp and -3779 bp/-1271 bp were greatly diminished. When the cDNA synthesized from using the gene-specific primer at -1128 bp (reverse) was subjected to RT-PCR analysis using the same reverse primer, paired with the forward primer at -3560 bp, no PCR product was observed (Fig.

Figure 3. Strand-specific cDNA and RT-PCR analysis of GnRH-E1 RNA.

A) Schematic diagram of the sense GnRH-E1 RNA variant, with a 3' polyA site located downstream of GnRH-E1 as predicted by RACE. PCR primers in reverse direction at -1128 bp or -1271 bp (reverse arrows) was used for strand-specific cDNA synthesis to capture the sense GnRH-E1 RNA. PCR analysis was performed using -1271 bp or -1128 bp reverse primer paired with the forward primers at -3560 bp, -3606 bp, -3779 bp, and -3746 bp (forward arrows) from the mouse *Gnrh1* TSS. Primer positions are aligned to the mouse conserved regulatory elements and coordinates diagrammed above. C) Strand-specific cDNA synthesized using -1271 bp reverse primer was subject to PCR analysis using the following primer pairs: -3560F/-1271R, -3606F/-1271R, -3779F/-1271R, -3746F/-1271R. Strand-specific cDNA synthesized using -1128 bp reverse primer was subject to PCR analysis using the primer pair -3560F/-1128R. D) Schematic diagram of the mouse antisense GnRH-E1 RNA variant, with a 3' polyA site predicted upstream of GnRH-E2 by RACE. PCR primers in forward direction at -3560 bp or -3371 bp (forward arrows) was used for strand-specific cDNA synthesis to capture the anti-sense GnRH-E1 RNA variant. PCR analysis was performed using -3371 bp or -3560 bp forward primer paired with the reverse primers at -1443 bp, -1271 bp, -1128 bp from the *Gnrh1* TSS. E) Strand-specific cDNA synthesized using -3371 bp forward primer was subject to PCR analysis using the following PCR primer pairs -3371F/-1443R, -3371F/-1271R, and -3371F/-1128R. Strand-specific cDNA synthesized using the -3560 bp forward primer was subject to PCR analysis using the primer pair at -3560F/-1443R. All reverse transcription reactions were performed on total RNA samples from GT1-7 cells with (+) and without (-) reverse transcriptase and were amplified by PCR in parallel with GT1-7 genomic DNA control and no-template control (NTC). The size of PCR amplicons is marked by 1 kbp DNA ladder.


A)
-4688
-4385
-3622
-3100
-2404
-2100
-278
-97
Gnrh1 TSS
E3
E2
E1
P
5' Start
Poly-A
-3560F
-3606F
-3746F
-3779F
-1128R
-1271R
B)
-1272R
-3560F
-3606F
-3779F
-3746F
-3560F/-1128R
DNA ladder
+ -1271R cDNA
- -1271R cDNA
Genomic DNA ctrl
NTC
+ -1128R cDNA
3.0 kb
2.0 kb
1.5 kb
1.0 kb
0.5 kb
C)
Poly-A
5' Start
-3560F
-3371F
-1128R
-1271R
-1443R
D)
-3371F
-1443R
-1271R
-1128R
-3560F/-1443R
+ -3371F cDNA
- -3371F cDNA
+ -3560F cDNA
- -3560F cDNA

3B). Reverse transcriptase reaction using GSP -1128 bp (reverse) failed to capture the 3' end of the sense GnRH-E1 RNA variant. Together, the data suggest that the transcription start site of the sense GnRH-E1 RNA variant resides between -3606 bp and -3746 bp, and that a 3' polyA termination site is located between -1271 bp and -1128 bp from the *Gnrh1* TSS.

Similarly, to capture the mouse antisense GnRH-E1 RNA variant, we used forward primers at -3560 bp and at -3371 bp for first-strand cDNA synthesis. The cDNA synthesized using gene-specific primer at -3371 bp (forward) was subject to RT-PCR analysis using the same forward primer, paired with reverse primers at -1128 bp, -1271 bp, and -1443 bp (Fig. 3C). PCR products of -3371 bp/-1443 bp and -3371bp/-1271 bp were successfully detected, though the PCR product of -3371 bp/-1271 bp was greatly diminished. The expected PCR product from -3371 bp/-1128 bp was not observed. When the cDNA synthesized from using the GSP at -3560 bp (forward) was subject to PCR analysis using the same forward primer, paired with the reverse primer at -1443 bp, no PCR product was observed (Fig. 3D). First-strand cDNA synthesis using GSP -3560 bp forward failed to capture the 3' end of the antisense GnRH-E1 RNA variant. Together, the data indicate that the TSS of the antisense GnRH-E1 RNA variant likely resides between -1443 bp and -1271 bp, and that a polyA termination site is located between -3560 bp and -3371 bp from the *Gnrh1* TSS.

Our strand-specific RT-PCR analyses of GnRH-E1 RNA indicate that sense and antisense variants are over 2 kb in length. We did not observe shorter PCR products within the -3746 bp to -1128 bp region, which suggests that GnRH-E1 RNA variants are not spliced. Analysis by Coding Potential Calculator (114) revealed that the -3746 bp/-1128 bp segment, and its reverse complementary strand, of the mouse *Gnrh1* gene

do not have coding potential. Furthermore, our data indicate that the sense GnRH-E1 RNA is transcribed from a distinct 5' start site located upstream of GnRH-E1, and the antisense GnRH-E1 RNA is transcribed from a start site located downstream of GnRH-E1. The 5' transcription start of sense and antisense GnRH-E1 RNA variants do not originate from a central transcription start site. Together, our data indicate that GnRH-E1 RNA variants are 5' capped, 3' polyadenylated RNA molecules of over 2 kb in length, and do not contain functional open reading frames (ORFs). These characteristics and the location of GnRH-E1 RNA transcription are consistent with the features of lincRNAs, a sub-category of lncRNAs transcribed from intergenic regions including enhancers. In addition, our approximated boundary of the sense and antisense GnRH-E1 RNAs between -1271 bp and -1128 bp from the *Gnrh1* TSS coincides with a decrease in the amount of RNA reads at approximately -1168 bp from the *Gnrh1* TSS.

GnRH-E1 RNA is a stable long intergenic noncoding RNA (lincRNA) localized in the nucleus

We then studied the localization and stability of GnRH-E1 RNA to gain clues about the functional role in *Gnrh1* gene regulation. RT-PCR analysis of nuclear and cytoplasmic extracts from GT1-7 neurons revealed that GnRH-E1 RNA is present in the nuclear RNA and absent from the cytoplasmic RNA. *Gnrh1* primary transcript (pre-mRNA) was observed in the nucleus, whereas *Gnrh1* mRNA was observed in both the nuclear and cytoplasmic extracts (Fig. 4). The localization of *Gnrh1* mRNA in both the nucleus and cytoplasm is consistent with the robust *Gnrh1* expression and GnRH synthesis in GT1-7 neurons. The predominantly nuclear localization of GnRH-E1 RNA suggests functional roles in the nucleus, such as the regulation of gene expression at

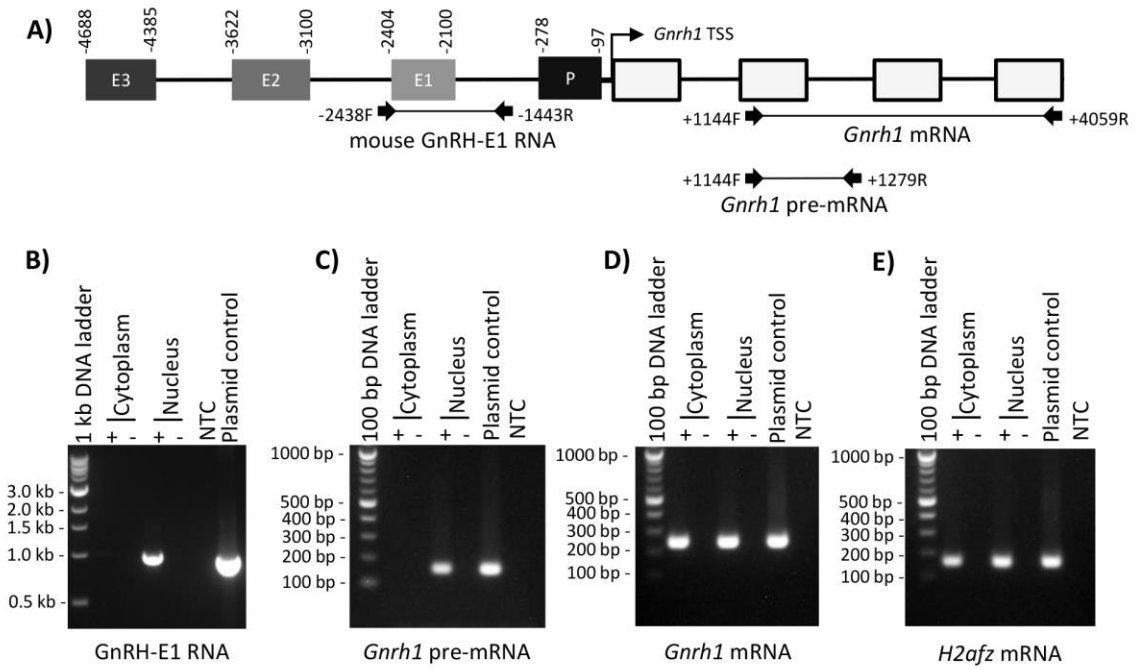


Figure 4. GnRH-E1 RNA is localized in the GT1-7 neuron nucleus.

A) Schematic diagram of the conserved regulatory elements upstream of the mouse *Gnrh1* TSS, which contains enhancers 1, 2, and 3 (E3, E2, E1, respectively), the promoter (P), and the *Gnrh1* gene with four exons (white boxes). Coordinates above the regulatory elements indicate positions with respect to the *Gnrh1* TSS. RT-PCR primers used in B-D are indicated by arrows, and expected PCR products are represented by a connecting line. Positions of PCR primers are aligned to the mouse conserved regulatory region diagrammed above. Nuclear and cytoplasmic extracts from GT1-7 neurons were analyzed for GnRH-E1 RNA (B), *Gnrh1* pre-mRNA (C), *Gnrh1* mRNA (D), and *H2afz* mRNA (E) by RT-PCR. RT-PCR analysis was performed on random hexamer-primed cDNA, where cDNA synthesized with (+) and without (-) reverse transcriptase were analyzed in parallel with plasmid DNA controls containing the respective PCR amplicons and no-template water control (NTC). The sizes of the PCR amplicons were marked by a 100 bp DNA ladder or a 1 kbp DNA ladder where indicated.

.

the level of transcription, rather than the participation in mRNA translation control in the cytoplasm.

LncRNAs are stable RNA molecules susceptible to siRNA knockdown and respond to cell signaling cascades (73, 79). In contrast, the enhancer-derived noncoding RNAs (enhancer RNAs or eRNAs) can be short-lived, with half-lives of minutes, though they also respond to cell signaling cascades (115). Our initial characterization of GnRH-E1 RNA showed that RNA expression from the *Gnrh1* gene regulatory region decreased in response to activation of the protein kinase-C signaling pathway following TPA treatment (45). We examined the stability of GnRH-E1 RNA, compared to *Gnrh1* mRNA and *Gnrh1* primary transcript. GT1-7 neurons were treated with 1 µg/mL of actinomycin D, a transcription inhibitor, or DMSO control. Total RNA was harvested from 2 hours to 24 hours following treatment (Fig. 5). RT-qPCR analysis revealed a decline in GnRH-E1 RNA abundance in actinomycin D-treated cells, with a 50% decrease in abundance between 8 hours and 24 hours after treatment (Fig. 5A). *Gnrh1* pre-mRNA showed a similar rate of decrease in abundance after treatment (Fig. 5B). *Gnrh1* pre-mRNA is likely continuously processed into mature *Gnrh1* mRNA. In contrast, *Gnrh1* mRNA was stable beyond 24 hours (Fig. 5C). DMSO-treated cells showed similar expression levels of GnRH-E1 RNA, *Gnrh1* mRNA and *Gnrh1* pre-mRNA up to 24 hours after treatment.

The knockdown of endogenous mouse GnRH-E1 RNA lowers Gnrh1 *gene expression*

To determine whether GnRH-E1 RNA functions in the regulation of *Gnrh1* gene expression, siRNA knockdown of the endogenous mouse GnRH-E1 RNA was performed in GT1-7 neurons, followed by RT-qPCR assay of *Gnrh1* mRNA and *Gnrh1* pre-mRNA expression. We utilized a custom designed siRNA duplex targeting a common region of

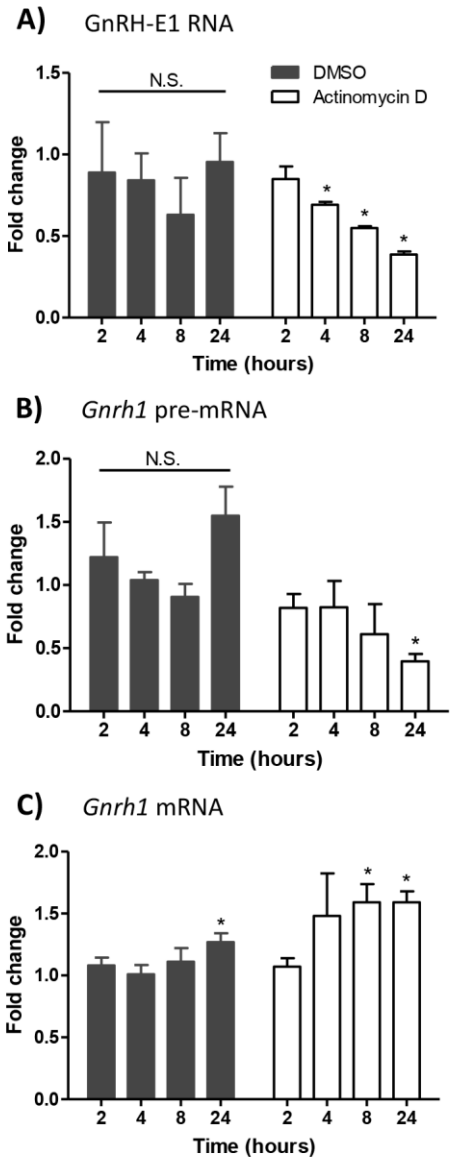


Figure 5. GnRH-E1 RNA, *Gnrh1* mRNA, and *Gnrh1* pre-mRNA stability in GT1-7 neurons.

A-C) GT1-7 neurons were treated with either 0.01% DMSO control (black bars) or 1 µg/mL actinomycin D (white bars). Total RNA was harvest at 2 hours to 24 hours after treatment. RT-qPCR analysis was performed to determine changes in GnRH-E1 RNA (A), *Gnrh1* pre-mRNA (B) and *Gnrh1* mRNA (C) expression. Relative expression is normalized to *H2afz* mRNA control. Data are displayed as the fold change from untreated cells that were harvested at the time of treatment, and as the mean ± SD. Statistical significance was determined by one-way ANOVA, followed by *post hoc* Tukey-Kramer HSD, where asterisks indicate statistical significance at $p<0.05$.

both the mouse sense and antisense GnRH-E1 RNA variant (though not the rat GnRH-E1 RNA expressed from the transgene in GT1-7 cells). At 36 hours after siRNA transfection, we observed a small, not statistically significant, decrease in GnRH-E1 RNA expression, compared to cells treated with negative control siRNA. GnRH-E1 RNA knockdown was more robust at 48 hours and 72 hours after siRNA transfection (Fig. 6A). Despite only a modest decrease in GnRH-E1 RNA at 36 hours after siRNA transfection, *Gnrh1* pre-mRNA expression significantly decreased at 36 hours in cells treated with siRNA targeting GnRH-E1 RNA, compared to cells treated with negative siRNA control. However, *Gnrh1* pre-mRNA levels were not affected by siRNA knockdown of GnRH-E1 RNA at 48 hours and 72 hours (Fig. 6B). Importantly, *Gnrh1* mRNA levels were markedly lowered at 72 hours after GnRH-E1 RNA knockdown (Fig. 6C). *Gnrh1* mRNA levels were not different between siRNA-treated cells and control-treated cells at 36 hours and 48 hours after siRNA transfection. The effect of GnRH-E1 RNA knockdown on both *Gnrh1* mRNA and *Gnrh1* pre-mRNA suggests that GnRH-E1 RNA is functionally significant in the regulation of *Gnrh1* expression at the level of transcription.

Over-expression of GnRH-E1 RNA in GT1-7 cells down-regulates Gnrh1 *transcriptional activity*

The robust correlation between GnRH-E1 RNA and *Gnrh1* mRNA expression in GT1-7 neurons, as well as the marked decrease in *Gnrh1* gene expression after GnRH-E1 RNA knockdown in GT1-7 neurons may suggest that GnRH-E1 RNA is a facilitator of *Gnrh1* gene expression. Because both the rat and mouse GnRH-E1 RNA are transcribed in GT1-7 neurons, we then tested whether the over-expression of the rat or

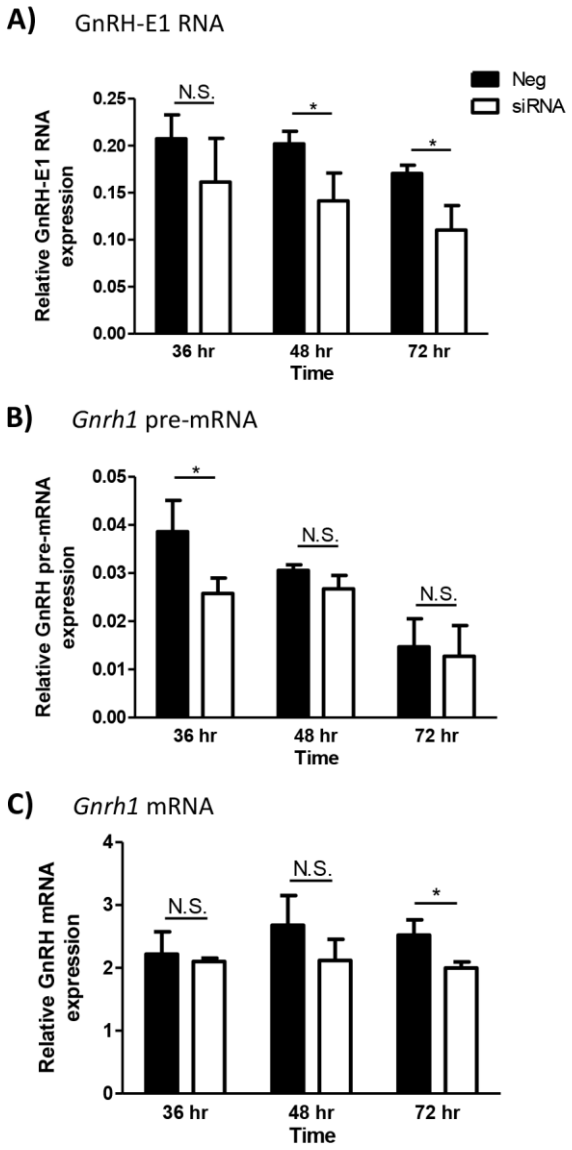


Figure 6. Effect of GnRH-E1 RNA knockdown on *Gnrh1* gene expression.

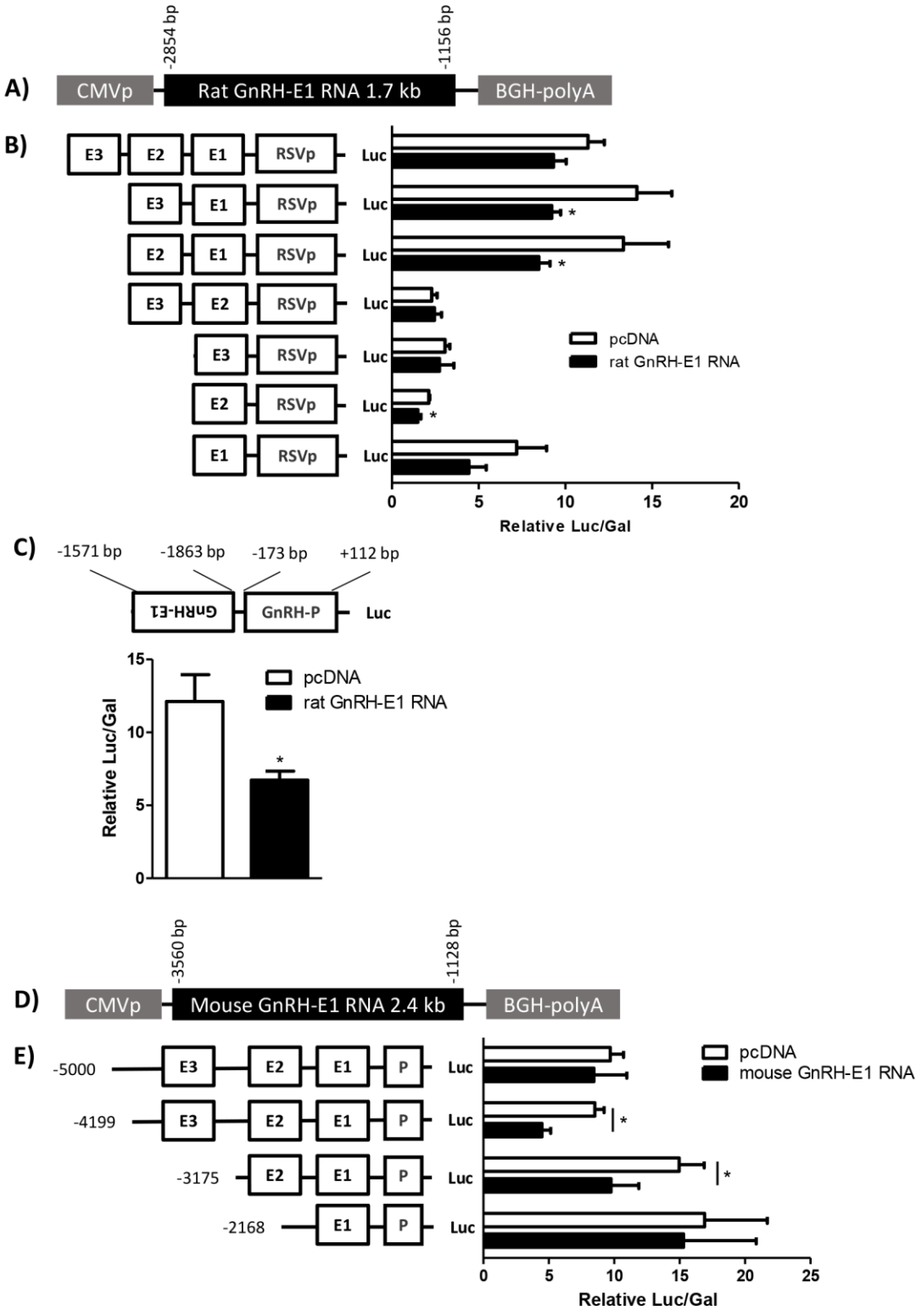
A-C) GT1-7 neurons were transfected with either negative siRNA control (Neg; black bars) or siRNA targeting both strands of the mouse GnRH-E1 RNA (siRNA; white bars). Total RNA was harvested at 36 hours, 48 hours, and 72 hours after siRNA transfection. RT-qPCR analysis was performed to determine GnRH-E1 RNA (A), *Gnrh1* pre-mRNA (B) and *Gnrh1* mRNA (C) expression. Relative RNA expression is normalized to *H2afz* mRNA expression at each time point, which is not affected by GnRH-E1 RNA siRNA knockdown. Data are displayed as the mean ± SD, where statistical significance was determined by Student's t-test compared between negative control and siRNA treatment at each time point. Asterisk indicates statistical significance, where $p<0.05$.

mouse GnRH-E1 RNA can affect *Gnrh1* gene transcriptional activity. We also asked whether GnRH-E1 RNA can act in *trans* and whether GnRH-E1 RNA action requires specific elements or location in the *Gnrh1* regulatory region. We used reporter plasmids plasmids carrying the well-characterized rat *Gnrh1* regulatory region to test the action of GnRH-E1 RNA on *Gnrh1* regulatory elements (30, 32, 33, 42).

We constructed an expression plasmid containing 1.7 kb (-2854 bp/-1156 bp) segment of the regulatory region located upstream of the rat *Gnrh1* TSS (Fig. 7A). Based on our preliminary data, transcription of the rat GnRH-E1 RNA from the rat transgene in GT1-7 neurons traverses *Gnrh1* enhancer 1, but does not overlap with the rat *Gnrh1* TSS. The 1.7 kb segment in our expression plasmid includes a significant portion, if not the full length, of the rat GnRH-E1 RNA from the transgene. Expression of the rat GnRH-E1 RNA, in the sense direction, is driven by a heterologous cytomegalovirus promoter (CMVp) and terminated by a plasmid-specific bovine growth hormone polyadenylation element (BGH-polyA). To test the action of the rat GnRH-E1 RNA on *Gnrh1* regulatory elements, we co-transfected the rat GnRH-E1 RNA expression plasmid with reporter plasmids containing only the conserved enhancers, GnRH-E3, E2, and E1, appended to a heterologous Rous sarcoma virus promoter (RSVp). In parallel, we co-transfected the rat GnRH-E1 RNA with reporter plasmids containing the combination of only two of the three enhancers, or reporter plasmids containing only one of the three enhancers alone, appended to the heterologous promoter. The over-expression of the rat GnRH-E1 RNA in GT17 neurons resulted in a marked decrease in the transcriptional activity of reporter plasmids containing E2 and E1 and that containing E3 and E1. Although the reporter plasmid containing E2 alone and a heterologous promoter showed relatively low activity when co-transfected with empty pcDNA expression vector, the over-expression of rat GnRH-E1 RNA resulted in a small,

Figure 7. Over-expression of the mouse and rat GnRH-E1 RNA in GT1-7 neurons.

A) Schematic diagram of expression plasmid carrying 1.7 kb (-2854 bp/-1156 bp) segment from upstream of rat *Gnrh1* TSS, cloned from the rat transgene in GT1-7 neurons, which contains the rat GnRH-E1 RNA and is integrated in the forward orientation. Expression of the rat GnRH-E1 RNA is driven by CMV promoter (CMVp) and terminated by a plasmid-specific 3' polyadenylation element (BGH-PolyA). B) GT1-7 neurons were transiently co-transfected with either empty pcDNA2.1 vector (white bars) or expression plasmid carrying the forward rat GnRH-E1 RNA (black bars), and luciferase reporter plasmid containing only the *Gnrh1* enhancers (E1, E2 and E3), combinations of two of the three, or one enhancer alone, upstream of the heterologous Rous sarcoma virus promoter (RSVp). C) GT1-7 neurons were transiently co-transfected with either empty pcDNA2.1 vector or expression plasmid carrying the rat GnRH-E1 RNA and luciferase reporter carrying the rat *Gnrh1* enhancer 1 (GnRH-E1) and promoter (GnRH-P), where GnRH-E1 is integrated in the reverse orientation. D) Schematic diagram of expression plasmid carrying 2.4 kb (-3560 bp/-1128 bp) segment from upstream of mouse *Gnrh1* TSS, which contains full-length mouse GnRH-E1 RNA and is integrated in the forward orientation. Expression of the sense GnRH-E1 RNA is driven by CMVp and terminated by a BGH-PolyA. E) GT1-7 neurons were transiently co-transfected with either empty pcDNA2.1 vector or expression plasmid carrying the forward mouse GnRH-E1 RNA, and luciferase reporter plasmid containing -5000 bp, -4199 bp, -3175 bp, and -2168 bp of the rat *Gnrh1* regulatory region. Each luciferase reporter plasmid contains the indicated *Gnrh1* enhancers (E1, E2 and E3) and *Gnrh1* promoter (P). Luciferase/β-galactosidase values were normalized to pGL3. Data are displayed as the mean ± SD. Asterisks indicate statistical significance determined using Student's T-test comparison between pcDNA-transfected and GnRH-E1 RNA transfected cells, where $p<0.05$.


A)
-2854 bp
-1156 bp
CMVp
Rat GnRH-E1 RNA 1.7 kb
BGH-polyA
B)
E3
E2
E1
RSVp
Luc
pcDNA
rat GnRH-E1 RNA
0
5
10
15
20
Relative Luc/Gal
C)
-1571 bp
-1863 bp
-173 bp
+112 bp
GnRH-E1
GnRH-P
Luc
Relative Luc/Gal
D)
-3560 bp
-1128 bp
CMVp
Mouse GnRH-E1 RNA 2.4 kb
BGH-polyA
E)
-5000
-4199
-3175
-2168
P
mouse GnRH-E1 RNA
25
Relative Luc/Gal

but significant decrease in E2 transcriptional activity (Fig. 7B). These observations indicate that the over-expression of the rat GnRH-E1 RNA down-regulates the activity of *Gnrh1* regulatory elements in GT1-7 neurons, where *Gnrh1* transcriptional activity is normally high, but the effects require the combination of E1 and either E2 or E3.

When we co-transfected the rat GnRH-E1 RNA expression plasmid with a reporter plasmid containing only the rat *Gnrh1* enhancer 1 and the promoter, the over-expression of the rat GnRH-E1 RNA also down-regulated the transcriptional activity of GnRH-E1 and GnRH-P (Fig. 7C). Together, these data suggest that the rat GnRH-E1 RNA can act in *trans* and show that the rat GnRH-E1 RNA can down-regulate the transcriptional activity of rat *Gnrh1* regulatory elements. Furthermore, the action of the rat GnRH-E1 RNA may require critical conserved regulatory elements: the combination of GnRH-E1 and either E2 or E3 if the enhancers are appended to a heterologous promoter, or *Gnrh1* enhancer 1 and the promoter alone.

To test the effect of the mouse GnRH-E1 RNA overexpression in GT1-7 neurons, we constructed an expression plasmid carrying a genomic segment from -3560 bp to -1128 bp upstream of the mouse *Gnrh1* TSS, a segment that contains the full length GnRH-E1 RNA, integrated in the forward orientation. Expression of the sense GnRH-E1 RNA from the plasmid is driven by a heterologous promoter, CMVp, and terminated by a heterologous 3’ polyadenylation element, BGH-polyA (Fig. 7D). In GT1-7 neurons, we transiently co-transfected the mouse sense GnRH-E1 RNA expression plasmid and luciferase reporter plasmids containing -5000 bp of the rat *Gnrh1* regulatory region and truncated reporter constructs containing -4199 bp, -3175 bp, and -2168 bp of the rat *Gnrh1* regulatory region. Over-expression of the mouse sense GnRH-E1 RNA did not significantly affect the transcriptional activity of reporter constructs containing -5000 bp

of the rat *Gnrh1* regulatory region, but caused a significant decrease in the transcriptional activity of the truncated reporter constructs containing -4199 bp and -3175 bp of the rat *Gnrh1* regulatory region. Over-expression of the mouse sense GnRH-E1 RNA did not affect the activity of the reporter plasmid containing -2168 bp of the rat *Gnrh1* regulatory region, which contains only *Gnrh1* enhancer 1 and promoter (Fig. 7E). The observation that of the mouse GnRH-E1 RNA can act in *trans* and affect the transcriptional activity of rat *Gnrh1* regulatory elements reflects the high level of evolutionary conservation between the rat and the mouse *Gnrh1* upstream region. The data also indicate that the action of the mouse GnRH-E1 RNA requires critical regions contained within -4199 bp and -3175 bp in the reporter plasmids.

As is the case for some lncRNAs, we hypothesized that GnRH-E1 RNA may interact with transcription factors that activate *Gnrh1* and act as a co-activator of *Gnrh1* transcription. If so, the over-expression of GnRH-E1 RNA in GT1-7 neurons may cause a sequestration of transcription factors or co-activators that act on the *Gnrh1* regulatory elements, thus resulting in an overall decrease in transcriptional activity. Alternatively, the over-abundance of GnRH-E1 RNA may cause interference with the action of endogenous GnRH-E1 RNA. Since both the sense and antisense strands of GnRH-E1 RNA are expressed in GT1-7 cells, it is possible that the over-expression of either the sense or antisense GnRH-E1 RNA may form a duplex with the complementary endogenous GnRH-E1 strand, thus inhibiting endogenous GnRH-E1 RNA action.

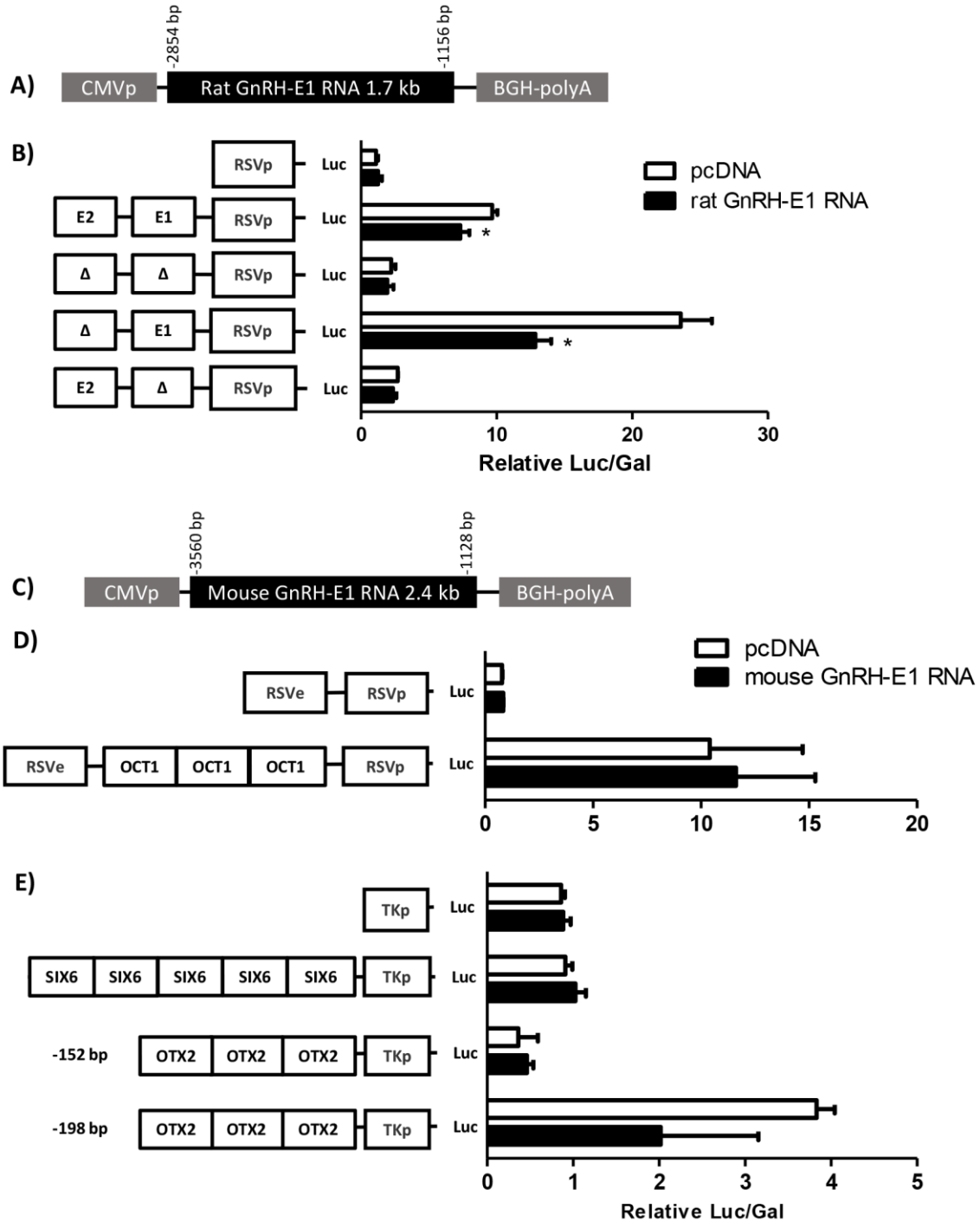
Our data showed that the combination of GnRH-E1 and either E2 or E3 is required for the GnRH-E1 RNA down-regulation of the transcriptional activity of *Gnrh1* regulatory elements in GT1-7 neurons. We asked whether this effect of GnRH-E1 RNA on the rat *Gnrh1* enhancer elements requires intact transcription factor binding sites in

GnRH-E2 and GnRH-E1. Octamer-binding transcription factor 1 (OCT1) is inducer of *Gnrh1* transcription, and has been shown to physically and functionally interact with nuclear factor 1 (NF1). OCT1/NF1 consensus sequences are found in the rat GnRH-E1 and GnRH-E2, where mutations in the OCT1/NF1 binding sites in either or both enhancers can lower the activity of the enhancers (112). In GT1-7 neurons, we transfected the rat GnRH-E1 RNA expression plasmid for over-expression (Fig. 8A), and co-transfected luciferase reporter plasmids containing rat GnRH E2 and E1 with mutated OCT1 consensus sequences in either or both E2 and E1. The over-expression of the rat GnRH-E1 RNA down-regulated the activity of the luciferase plasmid containing both intact GnRH-E2 and E1, which is consistent with our previous study. Mutations in OCT1 binding sites in both E2 and E1, or E1 alone, showed significantly diminished activity; GnRH-E1 RNA over-expression did not affect the activity of these reporter plasmids, compared to cells treated with the empty expression vector. Interestingly, the rat GnRH-E1 RNA down-regulation of enhancer activity requires GnRH-E1 with intact OCT1 binding sites (Fig. 8B). The over-expression of rat GnRH-E1 RNA can potentially interfere with the action of plasmid-transcribed GnRH-E1 RNA, resulting in a decrease in enhancer activity. If so, the data also suggest that the intact OCT1 binding site in GnRH-E1 is required for the transcription of GnRH-E1 RNA from the transfected plasmid.

Alternatively, the over-expression of rat GnRH-E1 RNA may cause a sequestration of OCT1 away from the binding sites contained in the transfected plasmid, thus decreasing the enhancer activity of GnRH-E1. We investigated this possibility when we transiently transfected the mouse GnRH-E1 RNA expression plasmid for over-expression in GT1-7 cells (Fig. 8C), and co-transfected a luciferase reporter plasmid carrying a multimer of the OCT1 consensus sequence (113). However, the over-

Figure 8. GnRH-E1 RNA does not act directly on transcription factor consensus sequences in GT1-7 neurons.

A) Schematic diagram of the expression plasmid carrying the 1.7 kb (-2854 bp/-1156 bp) rat GnRH-E1 RNA. B) GT1-7 neurons were transiently co-transfected with either empty pcDNA2.1 vector or expression plasmid carrying the rat GnRH-E1 RNA, and luciferase reporter plasmids carrying GnRH-E2 and GnRH-E1, or GnRH-E2 and E1 containing mutated OCT1 binding sites in either or both enhancer(s), upstream of a heterologous promoter, RSVp. Mutated OCT1 binding sites are represented by the delta (∆) symbol in E2 and E1. C) Schematic diagram of the expression plasmid carrying the 2.4 kb (-3560 bp/-1128 bp) mouse GnRH-E1 RNA. GT1-7 neurons were transiently transfected with either empty pcDNA 2.1 vector or the expression plasmid carrying the mouse GnRH-E1 RNA for over-expression (D-E). D) GT1-7 cells were co-transfected with luciferase reporter plasmids carrying a multimer of OCT1 consensus sequence, integrated between heterologous enhancer (RSVe) and heterologous promoter (RSVp), or RSVe/RSVp control reporter plasmid. E) GT1-7 cells were co-transfected with luciferase reporter plasmids carrying a multimer of SIX6 consensus sequence, the -152 bp OTX2 consensus sequence, or the -198 bp OTX2 consensus sequence, appended upstream of a heterologous thymidine kinase promoter (TKp). Luciferase/β-galactosidase values were normalized to pGL3. Data are displayed as the mean ± SD. Asterisks indicate statistical significance determined using Student's T-test comparison between pcDNA-transfected and GnRH-E1 RNA transfected cells, where $p<0.05$.


A)
-2854 bp
-1156 bp
CMVp
Rat GnRH-E1 RNA 1.7 kb
BGH-polyA
B)
RSVp
Luc
E2
E1
Δ
pcDNA
rat GnRH-E1 RNA
*
0
10
20
30
Relative Luc/Gal
C)
-3560 bp
-1128 bp
CMVp
Mouse GnRH-E1 RNA 2.4 kb
BGH-polyA
D)
RSVe
OCT1
pcDNA
mouse GnRH-E1 RNA
0
5
10
15
20
E)
TKp
SIX6
-152 bp
-198 bp
OTX2
0
1
2
3
4
5
Relative Luc/Gal

expression of the mouse GnRH-E1 RNA did not affect the activity of the reporter plasmid carrying OCT1 binding sites (Fig. 8D).

Additionally, we tested two other transcription factors that are known to regulate *Gnrh1* transcription to determine whether GnRH-E1 RNA indeed recruits transcription factors to their binding sites in the *Gnrh1* regulatory region. Homeodomain protein SIX6 binds at both the rat *Gnrh1* enhancer and promoter (43), and OTX2 binds at rat *Gnrh1* promoter (116). Both SIX6 and OTX2 are strong inducers of *Gnrh1* transcription, and are highly enriched specifically in GT1-7 cells. In GT1-7 cells transfected with the mouse GnRH-E1 RNA for over-expression, we co-transfected a luciferase reporter plasmid carrying a multimer of the SIX6 consensus sequence appended to a heterologous thymidine kinase promoter (TKp). In parallel, we co-transfected reporter plasmids carrying a multimer of the OTX2 consensus sequence found at -152 bp of the rat *Gnrh1* TSS, and a multimer of the OTX2 consensus at -198 bp of the rat *Gnrh1* TSS. However, the over-expression of GnRH-E1 RNA did not affect the activity of SIX6 or OTX2 on their binding sites, compared to cells that were treated with an empty expression vector (Fig. 8E). The transcriptional activity of the multimer of OTX2 binding sites at -198 bp of the rat *Gnrh1* showed a marked, but not statistically significant, decrease in cells transfected with mouse GnRH-E1 RNA. The data indicate that GnRH-E1 RNA does not recruit SIX6 and OTX2 directly to their binding sites alone.

Over-expression of GnRH-E1 RNA in GN11 cells up-regulates Gnrh1 *transcriptional activity*

In contrast to GT1-7 neurons, *Gnrh1* gene transcription is very low in GN11 cells, which do not express GnRH mRNA or GnRH-E1 RNA. We hypothesized that, if GnRH-

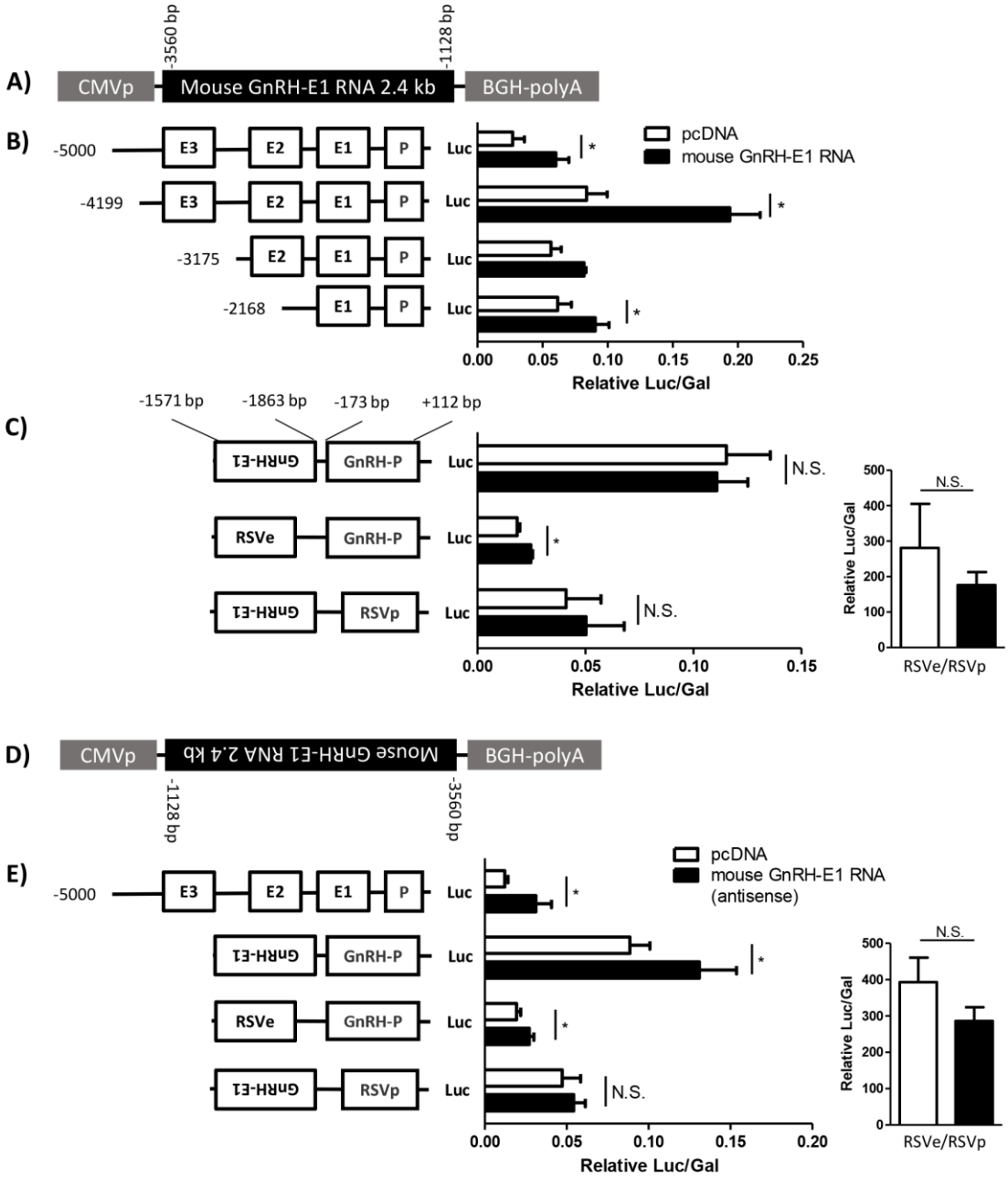
E1 RNA acts as a facilitator of *Gnrh1* gene expression, the over-expression of GnRH-E1 RNA in GN11 neurons may activate *Gnrh1* gene transcriptional activity. In GN11 cells in culture, we transiently co-transfected the mouse sense GnRH-E1 RNA expression plasmid (Fig. 9A) and luciferase reporter plasmids containing -5000 bp of the rat *Gnrh1* regulatory region and truncated reporter constructs containing -4199 bp, -3175 bp, and -2168 bp of the rat *Gnrh1* regulatory region. Over-expression of the mouse GnRH-E1 RNA induced transcriptional activity of the co-transfected *Gnrh1* gene regulatory elements, including -5000 bp, -4199 bp, -3175 bp, and -2168 bp of the rat *Gnrh1* gene regulatory region (Fig. 9B).

We also asked whether the over-expression of the mouse sense GnRH-E1 RNA can activate the transcriptional activity of the *Gnrh1* enhancer and/or the promoter. In GN11 cells, we transfected luciferase reporter plasmids carrying the rat *Gnrh1* enhancer 1 and promoter (GnRH-E1/GnRH-P), the heterologous RSV enhancer and the rat *Gnrh1* promoter (RSVe/GnRH-P), the rat GnRH-E1 and the heterologous RSV promoter (GnRH-E1/RSVp), or RSVe/RSVp control. The over-expression of the mouse sense GnRH-E1 RNA induced the activity of the *Gnrh1* promoter appended to RSVe, but did not affect the activity of GnRH-E1 appended to RSVp or the activity of the reporter plasmid carrying GnRH-E1 and GnRH-P alone. Over-expression of the mouse sense GnRH-E1 RNA did not affect the activity of the control luciferase plasmid carrying RSVe/RSVp, compared to cells that were transfected with empty pcDNA2.1 control expression plasmid (Fig. 9C).

We then tested whether the mouse antisense GnRH-E1 RNA also functions as a facilitator of *Gnrh1* transcriptional activity using an expression plasmid carrying the mouse GnRH-E1 RNA that is inserted in the reverse orientation, resulting in the CMV

Figure 9. Over-expression of the mouse GnRH-E1 RNA in GN11 cells.

A) A schematic diagram of the expression plasmid carrying the -3560 bp/-1128 bp segment from upstream of the mouse *Gnrh1* TSS, which contains the full-length mouse GnRH-E1 RNA that is integrated in the forward orientation. Expression of the sense GnRH-E1 RNA is driven by the CMV promoter (CMVp) and terminated by a plasmid-specific 3' polyadenylation element (BGH-PolyA). B-C) GN11 neurons were transiently co-transfected with either empty pcDNA2.1 vector (white bars) or expression plasmid carrying the forward mouse GnRH-E1 RNA (black bars) for over-expression and the luciferase reporter plasmids as indicated. B) GN11 cells were co-transfected with luciferase reporter plasmids containing -5000 bp, -4199 bp, -3175 bp, or -2168 bp of the rat *Gnrh1* regulatory region. Each luciferase reporter plasmid contains the indicated *Gnrh1* enhancers (E1, E2 and E3) and *Gnrh1* promoter (P). C) GN11 cells were co-transfected with luciferase plasmids carrying the rat *Gnrh1* enhancer 1 and promoter (GnRH-E1/GnRH-P), RSV enhancer and the rat *Gnrh1* promoter (RSVe/GnRH-P), the rat *Gnrh1* enhancer 1 and RSV promoter (GnRH-E1/RSVp), or RSVe/RSVp control (inset). For reporter plasmids carrying GnRH-E1, the rat *Gnrh1* enhancer is integrated in the reverse orientation in the plasmid. D) A schematic diagram of the expression plasmid carrying the full-length mouse GnRH-E1 RNA that is integrated in the reverse orientation, driven by CMVp and terminated by BGH-PolyA. E) The antisense GnRH-E1 RNA expression plasmid (black bars) or empty pcDNA2.1 expression plasmid (white bars) were transiently co-transfected with a reporter plasmid carrying the -5 kb rat *Gnrh1* gene regulatory region, GnRHE1/GnRH-P, GnRH-E1/RSVp, or RSVe/RSVp control (inset). Since RSVe/RSVp reporter expression is significantly higher than other reporter constructs, the relative luciferase/β-galactosidase values for RSVe/RSVp reporter are graphed separately for clarity in C and E. Luciferase/β-galactosidase values were normalized to pGL3. Data are displayed as the mean ± SD. Asterisks indicate statistical significance determined using Student's T-test comparison between pcDNA2.1-transfected and GnRH-E1 RNA-transfected cells, where $p<0.05$.


A)
-3560 bp
-1128 bp
CMVp
Mouse GnRH-E1 RNA 2.4 kb
BGH-polyA
B)
-5000
-4199
-3175
-2168
E3
E2
E1
P
Luc
pcDNA
mouse GnRH-E1 RNA
*
0.00
0.05
0.10
0.15
0.20
0.25
Relative Luc/Gal
C)
-1571 bp
-1863 bp
-173 bp
+112 bp
GnRH-E1
GnRH-P
RSVe
RSVp
N.S.
0.00
0.05
0.10
0.15
Relative Luc/Gal
Relative Luc/Gal
RSVe/RSVp
D)
Mouse GnRH-E1 RNA 2.4 kb
E)
mouse GnRH-E1 RNA
(antisense)

promoter-driven expression of the antisense GnRH-E1 RNA (Fig. 9D). Over-expression of the mouse antisense GnRH-E1 RNA induced the transcriptional activity of -5000 bp of the rat *Gnrh1* regulatory region, the transcriptional activity of the rat GnRH-E1 and GnRH-P alone, and the activity of RSVe/GnRH-P, but did not affect the activity of the GnRH-E1/RSVp reporter. The over-expression of the mouse antisense GnRH-E1 RNA did not affect the activity of the control RSVe/RSVp luciferase reporter plasmid, compared to cells that were transfected with empty pcDNA2.1 control expression plasmid (Fig. 9E).

Together, these results indicate that both the sense and antisense variants of the mouse GnRH-E1 RNA can induce the transcriptional activity of co-transfected rat *Gnrh1* regulatory elements in immature GnRH neurons, where the activity of *Gnrh1* regulatory elements is normally silent. The observation that either the sense or antisense GnRH-E1 RNA can induce the activity of the rat *Gnrh1* promoter, appended to a heterologous RSV enhancer suggests that GnRH-E1 RNA acts to induce *Gnrh1* transcriptional activity specifically by affecting *Gnrh1* promoter activity.

1.4 Discussion

The expression of GnRH-E1 RNA in GnRH cell models

Gonadotropin-releasing hormone gene expression and hormone secretion are highly specific functions of GnRH neurons in the hypothalamus. *Gnrh1* gene expression requires dynamic and specialized regulation by a number of molecular mechanisms. Previous studies in our laboratory first identified the expression of a *Gnrh1* enhancer-derived noncoding RNA (GnRH-E1 RNA) in the intergenic region of mouse *Gnrh1* and

Kctd9 by RT-PCR (45). Here, we characterize the mouse GnRH-E1 RNA as a 5' capped, 3' polyadenylated, long intergenic noncoding RNA of over 2 kb in length, with variants transcribed in the sense and antisense direction in the upstream regulatory region of the *Gnrh1* that appear not to be spliced. Expression of GnRH-E1 RNA correlates with *Gnrh1* mRNA expression in a mature GnRH neuron-specific manner. Our data indicate that GnRH-E1 RNA functions as an inducer of *Gnrh1* gene transcriptional activity.

Following a previous report from our lab that initially identified the expression of a noncoding RNA in the *Gnrh1* enhancer region, our study is the first to describe the physical and functional characteristics of the mouse GnRH-E1 RNA. RNA expression in the upstream intergenic region of the mouse or rat *Gnrh1* is not annotated in publicly available genome databases. However, human lincRNA RP11-395I14.2 (ENSG00000253476) has been identified and is transcribed from the intergenic region of human *GNRH1* and *KCTD9* in the same direction as *GNRH1* gene. Human lincRNA RP11-395I14.2 is 528 nt in length, is spliced, and contains a region in its exon 2 that is homologous to mouse GnRH-E1 RNA, but its function has yet to be described. While the human lincRNA RP11-395I14.2 expression profile is not exclusive to the brain, GnRH-E1 RNA expression in model cell lines, in contrast, suggests a hypothalamic cell-type specific expression pattern. Further investigation is required to examine whether human lincRNA RP11-395I14.2 and mouse GnRH-E1 RNA are functionally similar.

Both the sense and antisense variants of the mouse GnRH-E1 RNA were detected from strand-specific RT-PCR analysis. Although alternate transcription start and termination sites were indicated by RACE analysis, which also suggested expression of GnRH-E1 RNA variants of different lengths, strand-specific RT-PCR

analysis revealed GnRH-E1 RNA variants of over 2 kb in length. Our amplification of shorter GnRH-E1 RNA variants within this 2 kb region from -3606 bp to -1128 bp upstream of the *Gnrh1* TSS using conventional PCR consistently produced full-size amplicons, in parallel with full-size PCR amplicons from genomic DNA positive control template. This observation indicates that GnRH-E1 RNA variants do not undergo splicing within the region between -3606 bp and -1128 bp upstream of the *Gnrh1* TSS. It is possible, however, that shorter RNA transcripts are also generated within -3606 bp and -1128 bp, as suggested by the appearance of RNAs ranging from 700 nt to 3000 nt in our northern blot analysis. Additional investigation is required to address whether shorter noncoding RNAs are transcribed, as well as the functional significance of shorter enhancer-derived noncoding RNAs.

Strand-specific RT-PCR results suggest that the antisense GnRH-E1 RNA variant consists of rather discreet 5' and 3' ends. The sense GnRH-E1 RNA variant most likely contains a distinct 3' end between -1443 bp and -1128 bp upstream of the *Gnrh1* TSS. The 5' start site for GnRH-E1 RNA sense variant overlaps with the 3' UTR of the *Kctd9* gene located upstream of *Gnrh1*. From our RNA sequencing analysis, a small decrease in RNA reads is observed that aligns to the center of GnRH-E1, but our strand-specific RT-PCR experiments showed that unlike small enhancer RNAs (eRNAs), the 5' transcription start sites of the sense and antisense GnRH-E1 RNA variants do not originate from a central region of the enhancer (50, 61, 115, 117). The 5' transcription start sites reside in two separate locations at the 5' region of GnRH-E2 and downstream of GnRH-E1. Together, the physical characteristics of GnRH-E1 RNA are consistent with those of lncRNA and lincRNA, rather than eRNA.

Furthermore, the stability of GnRH-E1 RNA is consistent with the characteristics of lncRNA, rather than eRNA. LncRNAs can be stable RNA molecules that are susceptible to transient knockdown by siRNA and provide sustained regulation of gene expression (79). In contrast, eRNAs are rather short lived, with half-lives of minutes (52, 54, 62, 115). The stability of *Gnrh1* mRNA is evidently maintained for more than 24 hours, whereas the stabilities of GnRH-E1 RNA and *Gnrh1* pre-mRNA are comparatively shorter, with half-lives of about 8 hours. Nevertheless, we predicted that GnRH-E1 RNA is sufficiently stable for transient siRNA knockdown. In addition, the observation that GnRH-E1 RNA resides in the nucleus provides an important insight into the function of GnRH-E1 RNA as potential regulator of *Gnrh1* gene expression at the level of transcription.

The function of GnRH-E1 RNA in Gnrh1 *gene regulation*

To study the function of GnRH-E1 RNA in *Gnrh1* gene regulation, we tested the effect of mouse GnRH-E1 RNA knockdown in GT1-7 cells, and the effect of mouse or rat GnRH-E1 RNA over-expression in both GT1-7 and GN11 cell models. siRNA knockdown of the mouse GnRH-E1 RNA resulted in a decrease in *Gnrh1* expression, whereas over-expression of the mouse GnRH-E1 RNA in GN11 neurons induced the transcriptional activity of *Gnrh1* regulatory elements. Overall, the data indicate that GnRH-E1 RNA functions as a transcriptional inducer of *Gnrh1* gene expression.

For siRNA knockdown of GnRH-E1 RNA, we utilized a custom-designed siRNA duplex targeting a region that is common in both the sense and antisense mouse GnRH-E1 RNA. A significant knockdown of endogenous GnRH-E1 RNA was observed in GT1-7 neurons, accompanied by a decrease in *Gnrh1* pre-mRNA at 36 hours and a decrease

in *Gnrh1* mRNA at 72 hours after siRNA treatment. The delayed decrease in *Gnrh1* mRNA levels may reflect the long half-life (> 24 hours) and stability of *Gnrh1* mRNA (Fig. 5C). It is also important to note that our siRNA specifically targets only the mouse GnRH-E1 RNA, and does not target the rat GnRH-E1 RNA expressed from the rat transgene. The action of the rat GnRH-E1 RNA is likely to maintain *Gnrh1* expression during the siRNA knockdown of the mouse GnRH-E1 RNA. Even so, the knockdown of only the mouse GnRH-E1 RNA caused a down-regulation of both *Gnrh1* mRNA and the *Gnrh1* primary transcript. These observations provide evidence that GnRH-E1 RNA is a facilitator of *Gnrh1* gene expression.

Gnrh1 gene expression is tightly controlled by numerous mechanisms, including chromatin modifications and the action of transcription factors, which act on the enhancers and promoter upstream of *Gnrh1*. The effect of GnRH-E1 RNA knockdown on *Gnrh1* mRNA and *Gnrh1* pre-mRNA in GT1-7 cells may be a reflection of the number of mechanisms, in addition to GnRH-E1 RNA action, that regulate *Gnrh1* gene expression. A number of transcription factors and homeodomain proteins have been shown to bind at the rat *Gnrh1* enhancer region. Homeodomain proteins OCT1 and PREP1/PBX bind at the *Gnrh1* regulatory region, which specify *Gnrh1* expression in GT1-7 neurons (35-37). Temporal regulation of *Gnrh1* gene expression during GnRH neuron migration in development (40) is attributed to the zinc finger protein GATA-4 binding at the rat *Gnrh1* regulatory region (-1571 bp to -1863 bp) (38, 39). Homeodomain transcription factors MSX and DLX bind directly to CAATTA repeat elements in both GnRH-E1 and the promoter, where MSX1 or 2 represses *Gnrh1* promoter activity, while DLX2 or 5 relieves the repression. MSX and DLX dynamic control of *Gnrh1* expression is critical for specifying spatial distribution of GnRH neurons throughout development (41, 42). In addition, transcription factors SIX6 and VAX1 act as

inducers of *Gnrh1* expression, and are critical for maintaining *Gnrh1* expression during GnRH neuron maturation. However, VAX1 and SIX6 compete for occupation of the ATTA binding site in the *Gnrh1* enhancer and promoter (43, 44).

One possible mechanism of GnRH-E1 RNA function is through the interaction with transcription factors or chromatin remodelers that control *Gnrh1* expression. The decrease in GnRH-E1 RNA may lower the efficiency of transcription factor binding or chromatin modification at *Gnrh1* regulatory elements, but compensatory mechanisms and the other transcription factors may also play a role in maintaining *Gnrh1* transcription. In addition, GnRH-E1 RNA can act in *trans*, as evidenced by the effect of GnRH-E1 RNA overexpression on transfected *Gnrh1* regulatory elements. GnRH-E1 RNA may also act on distal genes, such as key transcription factors that regulate *Gnrh1* gene transcription, thus acting indirectly. In GT1-7 neurons, where *Gnrh1* gene expression is robust and controlled by numerous factors, GnRH-E1 RNA action may play only a limited role in facilitating the transcription of *Gnrh1* gene. Nevertheless, our data showed that the knockdown of the mouse GnRH-E1 RNA alone can result in a substantial decrease in endogenous *Gnrh1* gene expression.

Furthermore, the strength of GnRH-E1 RNA regulation of *Gnrh1* gene expression that we observed in our siRNA knockdown experiment is rather comparable to the vigor of lncRNA effects on individual target gene expression. Even among well-characterized lncRNAs described in the literature, the depletion of a lncRNA can generally down-regulate or remove repression of individual target gene expression (60, 69, 84, 118), but by and large does not completely abolish target gene expression. Even so, in these studies, changes lncRNA expression and the effect on their respective target gene(s) have been associated with changes in cell function, cell lineage commitment, and

changes in cell differentiation states. In animal studies of lncRNA function, changes in the expression of a lncRNA can amplify into changes in physiological states and pathology of disease (62, 77, 79, 119).

To firmly establish the function of GnRH-E1 RNA as an inducer of *Gnrh1* gene expression, we tested the effect of the mouse and rat GnRH-E1 RNA on the transcriptional activity of extensively studied and well-characterized rat *Gnrh1* regulatory elements (30, 32, 33, 42). When we co-transfected either the rat or the mouse GnRH-E1 RNA expression plasmid with reporter plasmids carrying the rat *Gnrh1* regulatory elements in GT1-7 neurons, we were surprised to find that the over-expression of GnRH-E1 RNA down-regulates the transcriptional activity of *Gnrh1* regulatory elements. However, the results indicate several important features of GnRH-E1 RNA function. First, GnRH-E1 RNA can act in *trans* and affect the transcriptional activity of co-transfected *Gnrh1* regulatory elements. Second, the effect of GnRH-E1 RNA action requires the presence of the combination of GnRH-E1 and either E2 or E3 when the enhancers are appended to a heterologous promoter. Third, the transcriptional activity of GnRH-E1 and *Gnrh1* promoter alone was down regulated by the over-expression of GnRH-E1 RNA. Together, these observations suggest critical regions of GnRH-E1 RNA action, and illustrate the functional relationship between the *Gnrh1* enhancer and promoter.

GnRH-E1 RNA abundance may be critical for maintaining the proper, balanced transcriptional activity of *Gnrh1*. It is important to note that GnRH-E1 RNA is expressed from both the endogenous mouse *Gnrh1* gene upstream region and the transgene containing 3 kb of the rat *Gnrh1* regulatory region in GT1-7 neurons. When we co-transfected either the mouse or the rat GnRH-E1 RNA expression plasmid with reporter

plasmids carrying *Gnrh1* regulatory elements, GnRH-E1 RNA is also expressed from the plasmids. In fact, when we transfected a reporter plasmid carrying GnRH-E1 and GnRH-P in GT1-7 cells, RT-PCR analysis using plasmid-specific primers captured the RNA transcribed from *Gnrh1* enhancer 1 contained in the transfected plasmid (data not shown). If GnRH-E1 RNA interacts with transcription factors that act as inducers of *Gnrh1* transcription, it is possible that an over-abundance of GnRH-E1 RNA levels in GT1-7 cells may sequester co-activators of *Gnrh1* gene transcription or interfere with the action of endogenous GnRH-E1 RNAs.

In GT1-7 cells, when we over-expressed the rat GnRH-E1 RNA and co-transfected reporter plasmids carrying GnRH-E2 and -E1 with mutations in either or both enhancer(s), the rat GnRH-E1 RNA down-regulation of enhancer activity requires GnRH-E1 with an intact OCT1 binding site. The data suggest that OCT1 binding at GnRH-E1 is required for the expression of GnRH-E1 RNA from the transfected plasmid and the over-abundance of the sense rat GnRH-E1 RNA interferes with the action the endogenous and plasmid-transcribed GnRH-E1 RNA. On the other hand, GnRH-E1 RNA may act to recruit transcription factors OCT1 and/or PBX to their binding site in GnRH-E1, and the over-abundance of GnRH-E1 RNA may cause a sequestration of transcription factors from *Gnrh1* enhancers, thus causing a down-regulation of enhancer activity. Alternatively, the presence of excess plasmid DNA with intact OCT1 sites may squelch expression from the reporter genes by competing for transcription factor binding independent of GnRH-E1 RNA.

In another set of experiments, we explored the possibility that GnRH-E1 RNA interacts with transcription factors, and functions as a co-activator of *Gnrh1* transcription by recruiting transcription factors to bind at the *Gnrh1* enhancer and promoter. The over-

expression of mouse GnRH-E1 RNA in GT1-7 cells did not affect the activity of luciferase reporter plasmids carrying multimers of OCT1, SIX6 or OTX2 consensus sequences. However, we are cautious to conclude that GnRH-E1 RNA does not interact with transcription factors. First, the failure of GnRH-E1 RNA to increase the activity of plasmids carrying multimers of transcription factor binding sites may be due to the fact that GnRH-E1 RNA requires context in addition to the consensus sequences alone or may be limited to acting on the *Gnrh1* promoter. Second, an over-expression of the transcription factors may be required for GnRH-E1 RNA to recruit these transcription factors to their binding sites on the transfected plasmid, to drive luciferase reporter activity. Conversely, the over-abundance of GnRH-E1 RNA may indeed cause a sequestration of transcription factors away from binding sites, but the sequestration has little effect due to the normally high levels of endogenous OCT1, SIX6 and OTX2 in GT1-7 cells. Third, the SIX6 consensus sequence can be competitively occupied by transcription factor VAX1, another inducer of *Gnrh1* transcription (44). Alternatively, it is possible that GnRH-E1 RNA interacts with more than one transcription factor or co-activator of *Gnrh1* gene transcription to serve as structural scaffolding for transcriptional co-activators (76). If so, the over-expression of GnRH-E1 RNA or the presence of excess enhancer DNA can cause sequestration of co-activators and thus prevent transcription factors from binding at the multimers (120, 121). Together, the data indicate that when we over-express the mouse or rat GnRH-E1 RNA, the decrease in the transcriptional activity of *Gnrh1* regulatory elements in GT1-7 neurons is most likely caused by the over-abundance of GnRH-E1 RNA and its interference with the actions of the endogenous GnRH-E1 RNA.

Indeed, the over-expression of either the sense or antisense mouse GnRH-E1 RNA in GN11 cells resulted in a substantial, up to 2-fold, increase in the transcriptional

activity of co-transfected rat *Gnrh1* regulatory elements. This observation not only demonstrates the high level of evolutionary conservation between the mouse and rat *Gnrh1* regulatory region but is also consistent with our characterization of GnRH-E1 RNA transcribed in both the sense and antisense direction. Importantly, the data indicate that GnRH-E1 RNA may indeed act as an inducer of *Gnrh1* gene transcription, and that both sense and antisense strands are functionally significant. GN11 neurons represent immature and migratory GnRH neurons, where *Gnrh1* mRNA is absent, and GnRH-E1 RNA expression is not detected. The transcriptional activity of transiently transfected *Gnrh1* regulatory elements is relatively silent in GN11 neurons, compared to the activity in GT1-7 neurons. The transcriptional activity of *Gnrh1* in GT1-7 neurons is 100-1000 times higher than that in GN11 neurons, as shown in the difference in RNA detection between GT1-7 cells and GN11 cells by RNA sequencing. Nevertheless, over-expression of GnRH-E1 RNA resulted in a marked increase in the transcriptional activity of *Gnrh1* regulatory elements. The data indicate that GnRH-E1 RNA is an inducer of *Gnrh1* gene transcription, thus may play a role in activating or de-repressing the transcriptional activity of *Gnrh1* gene regulatory elements in differentiating GnRH neurons.

Furthermore, we determined that the positive effect of GnRH-E1 RNA on *Gnrh1* regulatory elements requires the *Gnrh1* promoter, suggesting that GnRH-E1 RNA induction of *Gnrh1* transcription is through the induction of *Gnrh1* promoter activity. Interestingly, the mouse sense GnRH-E1 RNA induced the activity of the reporter plasmid containing the -2168 bp rat *Gnrh1* regulatory region which includes the enhancer and promoter, but did not affect the activity of the GnRH-E1/GnRH-P reporter. This observation may indicate that action of the sense GnRH-E1 RNA requires the genomic region between the enhancer and the promoter. On the other hand, the

antisense GnRH-E1 RNA induced the activity of GnRH-E1 and GnRH-P, as well as GnRH-P with a heterologous RSV enhancer, which provides strong indication that the GnRH-E1 RNA induces *Gnrh1* gene expression by inducing the activity of the *Gnrh1* promoter.

In this study, we established the mouse *Gnrh1* enhancer-derived noncoding RNA, GnRH-E1 RNA, as a facilitator of *Gnrh1* gene transcription in GnRH neuronal cell lines. Sense and antisense variants of GnRH-E1 RNA are 5' capped and 3' polyadenylated RNA molecules transcribed from the *Gnrh1* upstream regulatory region, but transcription evidently does not originate from a central region. The physical characteristics of GnRH-E1 RNA are consistent with those of lincRNAs. Our data indicate that GnRH-E1 RNA functions as an inducer of *Gnrh1* transcription by inducing the activity of the *Gnrh1* promoter, and may play a critical role in activating or de-repressing *Gnrh1* transcription during GnRH neuron maturation and development. Together, these data provide a foundation for future studies on the role of lincRNA in GnRH neuron function and maturation, with implications in reproductive neuroendocrinology.

1.5 Acknowledgements

The work described in part of this chapter has been submitted to the Public Library of Science (PLoS) One for publication. The following co-authors of the submitted manuscript gave their permission to include their contributions in this chapter: Brusman, Liza E., Iyer, Anita K., Webster, Nicholas J. G., and Mellon, Pamela L.

CHAPTER 2. Future Directions

The research described in this Dissertation characterized the physical and functional features of a novel noncoding RNA in the context of *Gnrh1* gene regulation. I investigated the expression profile of the mouse *Gnrh1* enhancer-derived noncoding RNA (GnRH-E1 RNA) in model cell lines of mature, differentiated and immature, migratory GnRH neurons. I investigated the genomic region from which the mouse GnRH-E1 RNA is transcribed, determined the 5' transcription start and 3' termination sites, and examined the strandedness of the GnRH-E1 RNA. This research established the functional significance of a novel noncoding RNA in *Gnrh1* gene regulation and set a foundation for future research to address additional questions about mechanisms and modalities of GnRH-E1 RNA action.

First, I showed RNA expression that overlaps the mouse *Gnrh1* enhancer 1 produces sense and antisense variants of a novel noncoding RNA. Mouse GnRH-E1 RNA variants are 5' capped, 3' polyadenylated RNA molecules of over 2 kb in length with no evident coding potential. Distinct 5' transcription start sites are located upstream and downstream of GnRH-E1 for the sense and antisense GnRH-E1 RNA variants, respectively. The data did not show evidence for splicing through the -3606 bp/-1128 bp segment, as we were not able to detect PCR amplicons that were shorter. However, it is possible that this full-length segment of GnRH-E1 RNA may be part of a larger transcript and may be spliced with another distal transcript. It is also possible that shorter RNAs are transcribed within the -3606 bp/-1128 bp region. We cannot rule out the possibility that the full-length GnRH-E1 RNAs of over 2 kb are the dominant transcripts or represent only a minority of RNAs transcribed from the *Gnrh1* enhancer region.

One possible experimental approach to define the full sequence of GnRH-E1 RNA is to use an RNA pull-down procedure to capture sense and antisense GnRH-E1 RNA variants. We may employ a method similar to chromatin isolation by RNA purification (ChIRP) (122), in which we design biotinylated DNA oligonucleotides targeting each strand of the mouse GnRH-E1 RNA to isolate GnRH-E1 RNA variants from GT1-7 neurons. Then, first- and second-strand cDNA may be synthesized from the isolated GnRH-E1 RNA, clone the double-stranded DNA products into a plasmid, such as TOPO, and verify the sequence of GnRH-E1 RNA variants by DNA sequencing. This experimental approach would also help determine whether shorter RNAs are transcribed from the *Gnrh1* enhancer region, and help confirm whether GnRH-E1 RNA variants are spliced.

The lack of annotations for GnRH-E1 RNA in mouse genome assemblies posed a challenge in defining the structure of GnRH-E1 RNA, but indicated that it is likely specific to the small population of GnRH neurons such that it would not be detected by sequencing RNA libraries of whole brain or hypothalamus. The RNA sequencing data clearly show RNA expression in the intergenic region of *Kctd9* and *Gnrh1* only in GT1-7 cells, suggesting hypothalamic GnRH neuron-specific expression of GnRH-E1 RNA. I expect that analysis of mouse hypothalamic tissue using double-label *in situ* hybridization for GnRH-E1 RNA and *Gnrh1* mRNA would show that GnRH-E1 RNA expression is co-localized with *Gnrh1* mRNA-expressing neurons.

A noncoding RNA transcript from the intergenic region between *GNRH1* and *KCTD9* in the human has been recorded. The human lincRNA RP11-395I14.2 (ENSG00000253476) shares a homologous region with the mouse GnRH-E1 RNA, but the expression profile of the human lincRNA does not appear to be exclusive to the

brain, while the mouse GnRH-E1 RNA expression is specific to a model cell line representing mature, differentiated hypothalamic GnRH neurons. Furthermore, the function of the human lincRNA is not known. One interesting and worthwhile future study would be to investigate whether the human lincRNA RP11-395I14.2 is functionally similar to GnRH-E1 RNA.

In the research described in this Dissertation, I concluded that GnRH-E1 RNA acts as an enhancer of *Gnrh1* transcription, in which both the sense and antisense variants of mouse GnRH-E1 RNA can play a role in activating or de-repressing the transcriptional activity of co-transfected rat *Gnrh1* regulatory elements in GN11 cells. I have discussed some of the additional, compensatory mechanisms that tightly control *Gnrh1* expression in mature GnRH neurons. We cannot rule out the possibility that GnRH-E1 RNA acts in *trans* in the regulation of a distal gene to indirectly control *Gnrh1* transcription. For example, *Evf2* is a noncoding RNA transcribed from the enhancer region of *Dlx-5-6* and acts in *trans* to increase the transcriptional activity of the homeodomain protein, Dlx-2. *Evf2* recruits DLX-2 as a co-activator to increase the activity of the *Dlx-5/6* enhancer (69, 71). Thus, the functional effect of a noncoding RNA can be *cis,* while the underlying mechanism is acting in *trans.* Alternatively, GnRH-E1 RNA may interact with chromatin modifiers that are involved in the regulation of *Gnrh1* transcription as well as the regulation of distal gene(s).

We tested whether the over-expression of the mouse GnRH-E1 RNA in GT1-7 cells can increase the activity of luciferase reporters carrying multimers of OCT1, SIX6 and OTX2 consensus sequences. Based on our assay, we concluded that GnRH-E1 RNA most likely does not interact with transcription factors. However, I have discussed the limitations of our assay, and it is possible for GnRH-E1 RNA to interact with other

modulators of *Gnrh1* transcription. It is also possible that GnRH-E1 RNA may interact with more than one transcription factor or chromatin remodeler that act on the *Gnrh1* regulatory region. GnRH-E1 RNA may serve as a co-factor for transcriptional modulators and provide structural scaffolding for transcription complexes. LincRNAs that participate in epigenetic remodeling complexes have been described to interact with multiple proteins, providing structural support for chromatin remodeling complexes at target genes (74, 76).

ChIRP or RNA immunoprecipitation (RIP) could be used to address whether GnRH-E1 RNA interacts with transcription factors and/or chromatin remodelers to modulate *Gnrh1* gene transcription. Since RNA pull-down efficiency can be hindered by the abundance of endogenous GnRH-E1 RNA in GT1-7 cells, we may employ an RNA pull-down method that has been previously described (72, 123) to overcome the limitation of isolating endogenous RNA with its potential binding partners. We can use our knowledge about the length and strandedness of GnRH-E1 RNA to construct an *in vitro*-transcribed, biotin-labeled RNA for a pull-down experiment to identify potential binding partners (72). We may test whether GnRH-E1 RNA interacts with transcription factors such as SIX6, OTX2, and OCT1 using RIP. We may also test whether GnRH-E1 RNA interacts with more than one transcription factor, or chromatin remodeling complexes, which would indicate that GnRH-E1 RNA may serve as a structural scaffold for transcriptional co-regulators. In addition, ChIRP can help identify proteins that physically associate with GnRH-E1 RNA, as well as the genomic location where GnRH-E1 RNA acts. If GnRH-E1 RNA modulates *Gnrh1* gene transcription indirectly, by modulating a distal gene that codes for a modulator of *Gnrh1* transcription, ChIRP may help identify additional genomic targets of GnRH-E1 RNA.

GnRH-E1 RNA participation in DNA looping interaction between the enhancer and the promoter is also a potential mechanism of action. In fact, kisspeptin activation of the kisspeptin response element in the upstream regulatory region of the mouse *Gnrh1* gene has been proposed to cause DNA looping interaction between the regulatory elements and the *Gnrh1* promoter. The participation of the transcription factor OTX2 has also been proposed as a critical modulator of *Gnrh1* enhancer-promoter interaction (124, 125). However, the kisspeptin regulation of *Gnrh1* gene transcriptional activity was observed in transfected plasmids. Our treatment of GT1-7 cells with kisspeptin did not produce significant change in endogenous *Gnrh1* mRNA (data not shown).

Nevertheless, lncRNAs transcribed from enhancers have been described to participate in DNA looping interaction between enhancers and promoters (60, 84, 126). It is also possible that GnRH-E1 RNA participates in enhancer-promoter interactions in response to cellular signals, not limited to kisspeptin, that regulate *Gnrh1* expression. Based on the observation that GnRH-E1 RNA knockdown decreased the expression of endogenous *Gnrh1* pre-mRNA and *Gnrh1* mRNA, we can use chromosome conformation assay (3C) after GnRH-E1 RNA knockdown to test whether the decrease in *Gnrh1* gene expression is due to changes in *Gnrh1* enhancer-promoter DNA looping.

Finally, the validation of GnRH-E1 RNA function *in vivo* has important implications in lncRNA function in reproductive physiology. The studies described in this Dissertation provide important information about the physical attributes of GnRH-E1 RNA, such as length, strandedness, polyadenylation, stability, and cellular localization, which we can use to design effective methods to stably knockdown GnRH-E1 RNA in the mouse. Intracerebroventricular (ICV) injection can be used to deliver a viral vector expressing shRNA that targets GnRH-E1 RNA for knockdown in the adult mouse. We

may then perform a fertility assessment to determine whether the decrease in GnRH-E1 RNA alters the timing of pubertal onset, time to first litter, litter size, and/or estrous cyclicity.

As mentioned in the Introduction of the Dissertation, mice carrying a 728 bp (-2806 bp/-2078 bp) deletion in *Gnrh1* enhancer 1 show lower *Gnrh1* mRNA expression in the brain, higher *Gnrh1* mRNA expression in the ovaries, and irregularities in estrous cyclicity (127). The deletion in *Gnrh1* enhancer knockout mice (GREKO) mice consists of a large portion of the GnRH-E1 RNA, a portion that is common to both the sense and antisense GnRH-E1 RNA variants. The GREKO mouse would serve as a valuable tool to study and validate the function of GnRH-E1 RNA in a rescue study performed *in vivo*. Based on the data described in this Dissertation, over-expression of GnRH-E1 RNA in GN11 cells resulted in a marked increase in the transcriptional activity of *Gnrh1* regulatory elements. We may administer exogenous GnRH-E1 RNA in GREKO mice and assess the reproductive capacity. If the administration of either the sense or antisense GnRH-E1 RNA can rescue the reproductive capacity of GREKO mice, the data could indicate the functional significance of either strand of GnRH-E1 RNA. Since the deletion does not contain the full length of GnRH-E1 RNA, we may also replace only the portion of GnRH-E1 RNA that is deleted in GREKO mice; the result would indicate a critical region for GnRH-E1 RNA function. On the other hand, GnRH-E1 RNA may not be sufficient to rescue the reproductive phenotype of GREKO mice, which would indicate that GnRH-E1 RNA plays only a limited role in the regulation of *Gnrh1* gene expression. Based on previous reports on the functional significance of the rat *Gnrh1* enhancer 1 discussed earlier, I expect that the deletion of the mouse enhancer would abolish the genomic context that is necessary for the complex transcriptional regulation of *Gnrh1* gene, regardless of the presence of GnRH-E1 RNA expression from GnRH-E1.

Functional validation of GnRH-E1 RNA action *in vivo* may carry implications in the clinical understanding of reproductive disorders associated with the dysregulation of *Gnrh1* expression.

CONCLUSIONS

After the initial discovery of an RNA transcribed from the *Gnrh1* enhancer, I have better defined the physical features and functional role of GnRH-E1 RNA in *Gnrh1* gene regulation. The work described in this Dissertation demonstrated GnRH-E1 RNA as an inducer of *Gnrh1* gene expression in cell models. This study provides foundational knowledge for future studies on the role of noncoding RNAs in GnRH neuron maturation, with implications in reproductive development and function.

Transcription at the *Gnrh1* upstream regulatory region overlaps two enhancers of the *Gnrh1* gene, producing sense and antisense variants of GnRH-E1 RNA that carry 5' capping structure and are 3' polyadenylation (polyA) tail. GnRH-E1 RNA is a relatively stable lncRNA that resides in the nucleus of immortalized, differentiated hypothalamic GnRH neurons. The physical characteristics of GnRH-E1 RNA are consistent with those of the lncRNA category.

The functional role of GnRH-E1 RNA in the regulation of *Gnrh1* gene expression is complex. I have concluded from the data that GnRH-E1 RNA acts as a facilitator of *Gnrh1* gene transcription. The knockdown of GnRH-E1 RNA in immortalized mature hypothalamic GnRH neurons resulted in a significant decrease in *Gnrh1* gene expression. The over-expression of either the sense or antisense GnRH-E1 RNA induced the transcriptional activity of transfected *Gnrh1* regulatory elements, suggesting that GnRH-E1 RNA is able to activate or de-repress *Gnrh1* gene transcription. In addition, the data showed that in mature, differentiated GnRH neurons where *Gnrh1* gene expression is robust, the abundance of GnRH-E1 RNA may be critical for maintaining the balance of *Gnrh1* transcriptional activity. These observations reflect the

complex underlying mechanisms of GnRH-E1 RNA function in the dynamic control of *Gnrh1* gene expression.

I established the functional significance of the novel *Gnrh1* enhancer-derived noncoding RNA in the regulation of *Gnrh1* expression. The data implicate GnRH-E1 RNA function as a potential regulator of reproductive development and function. Future studies should focus on the functional mechanisms of GnRH-E1 RNA action and validation of GnRH-E1 RNA function in animal studies. This study serves as a positive contribution to the wealth of knowledge about noncoding RNA function and a positive impact on our understanding of reproductive physiology.

REFERENCES

1. Tobet SA, Schwarting GA. Minireview: recent progress in gonadotropin-releasing hormone neuronal migration. Endocrinology. 2006;147(3):1159-65.

2. Oakley AE, Breen KM, Clarke IJ, Karsch FJ, Wagenmaker ER, Tilbrook AJ. Cortisol reduces gonadotropin-releasing hormone pulse frequency in follicular phase ewes: influence of ovarian steroids. Endocrinology. 2009;150(1):341-9.

3. Gore AC, Attardi B, DeFranco DB. Glucocorticoid repression of the reproductive axis: effects on GnRH and gonadotropin subunit mRNA levels. Mol Cell Endocrinol. 2006;256(1-2):40-8.

4. Breen KM, Davis TL, Doro LC, Nett TM, Oakley AE, Padmanabhan V, et al. Insight into the neuroendocrine site and cellular mechanism by which cortisol suppresses pituitary responsiveness to gonadotropin-releasing hormone. Endocrinology. 2008;149(2):767-73.

5. Chappell PE. Clocks and the black box: circadian influences on gonadotropin-releasing hormone secretion. J Neuroendocrinol. 2005;17(2):119-30.

6. Gore AC. Diurnal rhythmicity of gonadotropin-releasing hormone gene expression in the rat. Neuroendocrinology. 1998;68(4):257-63.

7. Chappell PE, White RS, Mellon PL. Circadian gene expression regulates pulsatile gonadotropin-releasing hormone (GnRH) secretory patterns in the hypothalamic GnRH-secreting GT1-7 cell line. J Neurosci. 2003;23(35):11202-13.

8. Gillespie JM, Chan BP, Roy D, Cai F, Belsham DD. Expression of circadian rhythm genes in gonadotropin-releasing hormone-secreting GT1-7 neurons. Endocrinology. 2003;144(12):5285-92.

9. Goldman BD. The circadian timing system and reproduction in mammals. Steroids. 1999;64(9):679-85.

10. Schneider JE. Energy balance and reproduction. Physiology & behavior. 2004;81(2):289-317.

11. Gore AC. Gonadotropin-releasing hormone (GnRH) neurons: gene expression and neuroanatomical studies. Prog Brain Res. 2002;141:193-208.

12. Gore AC, Roberts JL, Gibson MJ. Mechanisms for the regulation of gonadotropin-releasing hormone gene expression in the developing mouse. Endocrinology. 1999;140(5):2280-7.

13. Gore AC, Wu TJ, Rosenberg JJ, Roberts JL. Gonadotropin-releasing hormone and NMDA receptor gene expression and colocalization change during puberty in female rats. J Neurosci. 1996;16(17):5281-9.

14. Terasawa E, Fernandez DL. Neurobiological mechanisms of the onset of puberty in primates. Endocrine reviews. 2001;22(1):111-51.

15. Gore AC. Gonadotropin-releasing hormone neurons, NMDA receptors, and their regulation by steroid hormones across the reproductive life cycle. Brain Res Brain Res Rev. 2001;37(1-3):235-48.

16. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, et al. The GPR54 gene as a regulator of puberty. N Engl J Med. 2003;349(17):1614-27.

17. Mayer C, Boehm U. Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. Nat Neurosci. 2011;14(6):704-10.

18. Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA. Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. J Neurosci. 2009;29(38):11859-66.

19. Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CV, Jafarzadehshirazi MR, et al. Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology. 2007;148(12):5752-60.

20. Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, et al. Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. J Neurosci. 2010;30(8):3124-32.

21. Cariboni A, Maggi R, Parnavelas JG. From nose to fertility: the long migratory journey of gonadotropin-releasing hormone neurons. Trends Neurosci. 2007;30(12):638-44.

22. Tsai PS, Gill JC. Mechanisms of disease: Insights into X-linked and autosomal-dominant Kallmann syndrome. Nat Clin Pract Endocrinol Metab. 2006;2(3):160-71.

23. Kaiser UB, Kuohung W. KiSS-1 and GPR54 as new players in gonadotropin regulation and puberty. Endocrine. 2005;26(3):277-84.

24. Swaab DF. Prader-Willi syndrome and the hypothalamus. Acta Paediatr Suppl. 1997;423:50-4.

25. Layman LC. Genetic causes of human infertility. Endocrinol Metab Clin North Am. 2003;32(3):549-72.

26. Mellon PL, Wetsel WC, Windle JJ, Valenca MM, Goldsmith PC, Whyte DB, et al. Immortalized hypothalamic gonadotropin-releasing hormone neurons. Ciba Found Symp. 1992;168:104-17.

27. Radovick S, Wray S, Lee E, Nicols DK, Nakayama Y, Weintraub BD, et al. Migratory arrest of gonadotropin-releasing hormone neurons in transgenic mice. Proc Natl Acad Sci USA. 1991;88:3402-6.

28. Radovick S, Wray S, Lee E, Nicols DK, Nakayama Y, Weintraub BD, et al. Migratory arrest of gonadotropin-releasing hormone neurons in transgenic mice. Proc Natl Acad Sci USA. 1991;88(8):3402-6.

29. Iyer AK, Miller NL, Yip K, Tran BH, Mellon PL. Enhancers of GnRH transcription embedded in an upstream gene use homeodomain proteins to specify hypothalamic expression. Molecular endocrinology (Baltimore, Md. 2010;24:1949-64.

30. Nelson SB, Lawson MA, Kelley CG, Mellon PL. Neuron-specific expression of the rat gonadotropin-releasing hormone gene is conferred by interactions of a defined promoter element with the enhancer in GT1-7 cells. Mol Endocrinol. 2000;14(9):1509-22.

31. Iyer AK, Miller NL, Yip K, Tran BH, Mellon PL. Enhancers of GnRH transcription embedded in an upstream gene use homeodomain proteins to specify hypothalamic expression. Mol Endocrinol. 2010;24(10):1949-64.

32. Whyte DB, Lawson MA, Belsham DD, Eraly SA, Bond CT, Adelman JP, et al. A neuron-specific enhancer targets expression of the gonadotropin-releasing hormone gene to hypothalamic neurosecretory neurons. Mol Endocrinol. 1995;9(4):467-77.

33. Lawson MA, MacConell LA, Kim J, Powl BT, Nelson SB, Mellon PL. Neuron-specific expression In vivo by defined transcription regulatory elements of the gonadotropin-releasing hormone gene. Endocrinology. 2002;143:1404-12.

34. Novaira HJ, Yates M, Diaczok D, Kim H, Wolfe A, Radovick S. The gonadotropin-releasing hormone cell-specific element is required for normal puberty and estrous cyclicity. J Neurosci. 2011;31(9):3336-43.

35. Eraly SA, Nelson SB, Huang KM, Mellon PL. Oct-1 binds promoter elements required for transcription of the gonadotropin-releasing hormone gene. Molecular endocrinology (Baltimore, Md. 1998;12:469-81.

36. Rave-Harel N, Givens ML, Nelson SB, Duong HA, Coss D, Clark ME, et al. TALE homeodomain proteins regulate gonadotropin-releasing hormone gene expression independently and via interactions with Oct-1. The Journal of biological chemistry. 2004;279(29):30287-97.

37. Clark ME, Mellon PL. The POU homeodomain transcription factor Oct-1 is essential for activity of the gonadotropin-releasing hormone neuron-specific enhancer. Mol Cell Biol. 1995;15:6169-77.

38. Lawson MA, Buhain AR, Jovenal JC, Mellon PL. Multiple factors interacting at the GATA sites of the gonadotropin-releasing hormone neuron-specific enhancer regulate gene expression. Molecular endocrinology (Baltimore, Md. 1998;12:364-77.

39. Lawson MA, Whyte DB, Mellon PL. GATA factors are essential for activity of the neuron-specific enhancer of the gonadotropin-releasing hormone gene. Mol Cell Biol. 1996;16:3596-605.

40. Lawson MA, Mellon PL. Expression of GATA-4 in migrating GnRH neurons of the developing mouse. Mol Cell Endocrinol. 1998;140:157-61.

41. Givens ML, Rave-Harel N, Goonewardena VD, Kurotani R, Berdy SE, Swan CH, et al. Developmental regulation of gonadotropin-releasing hormone gene expression by the MSX and DLX homeodomain protein families. J Biol Chem. 2005;280(19):19156-65.

42. Kelley CG, Givens ML, Rave-Harel N, Nelson SB, Anderson S, Mellon PL. Neuron-restricted expression of the rat gonadotropin-releasing hormone gene is conferred by a cell-specific protein complex that binds repeated CAATT elements. Mol Endocrinol. 2002;16(11):2413-25.

43. Larder R, Clark DD, Miller NL, Mellon PL. Hypothalamic dysregulation and infertility in mice lacking the homeodomain protein Six6. J Neurosci. 2011;31(2):426-38.

44. Hoffmann HM, Trang C, Gong P, Kimura I, Pandolfi EC, Mellon PL. Deletion of Vax1 from GnRH neurons abolishes GnRH expression and leads to hypogonadism and infertility. J Neurosci. 2016;in press.

45. Iyer AK, Brayman MJ, Mellon PL. Dynamic chromatin modifications control GnRH gene expression during neuronal differentiation and protein kinase C signal transduction. Mol Endocrinol. 2011;25(3):460-73.

46. Ong CT, Corces VG. Enhancer function: new insights into the regulation of tissue-specific gene expression. Nat Rev Genet. 2011;12(4):283-93.

47. Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, Harp LF, et al. Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature. 2009;459(7243):108-12.

48. Bulger M, Groudine M. Functional and mechanistic diversity of distal transcription enhancers. Cell. 2011;144(3):327-39.

49. Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R, Willingham AT, et al. RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science (New York, NY). 2007;316(5830):1484-8.

50. Kim TK, Hemberg M, Gray JM, Costa AM, Bear DM, Wu J, et al. Widespread transcription at neuronal activity-regulated enhancers. Nature. 2010;465(7295):182-7.

51. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, et al. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet. 2007;39(3):311-8.

52. Natoli G, Andrau JC. Noncoding transcription at enhancers: general principles and functional models. Annu Rev Genet. 2012;46:1-19.

53. De Santa F, Barozzi I, Mietton F, Ghisletti S, Polletti S, Tusi BK, et al. A large fraction of extragenic RNA pol II transcription sites overlap enhancers. PLoS Biol. 2010;8(5):e1000384.

54. Koch F, Fenouil R, Gut M, Cauchy P, Albert TK, Zacarias-Cabeza J, et al. Transcription initiation platforms and GTF recruitment at tissue-specific enhancers and promoters. Nature structural & molecular biology. 2011;18(8):956-63.

55. Sun M, Kraus WL. From Discovery to Function: The Expanding Roles of Long Non-Coding RNAs in Physiology and Disease. Endocrine reviews. 2014:er20141034.

56. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet. 2009;10(3):155-9.

57. Jia H, Osak M, Bogu GK, Stanton LW, Johnson R, Lipovich L. Genome-wide computational identification and manual annotation of human long noncoding RNA genes. RNA (New York, NY). 2010;16(8):1478-87.

58. Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, et al. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res. 2012;22(9):1775-89.

59. Orom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, et al. Long noncoding RNAs with enhancer-like function in human cells. Cell. 2010;143(1):46-58.

60. Pnueli L, Rudnizky S, Yosefzon Y, Melamed P. RNA transcribed from a distal enhancer is required for activating the chromatin at the promoter of the gonadotropin alpha-subunit gene. Proc Natl Acad Sci U S A. 2015;112(14):4369-74.

61. Melgar MF, Collins FS, Sethupathy P. Discovery of active enhancers through bidirectional expression of short transcripts. Genome Biol. 2011;12(11):R113.

62. Lai F, Shiekhattar R. Enhancer RNAs: the new molecules of transcription. Curr Opin Genet Dev. 2014;25:38-42.

63. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, et al. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet. 2007;39(3):311-8.

64. Sun M, Kraus WL. From Discovery to Function: The Expanding Roles of Long Non-Coding RNAs in Physiology and Disease. Endocr Rev. 2015;in press:er00009999.

65. Melo CA, Drost J, Wijchers PJ, van de Werken H, de Wit E, Oude Vrielink JA, et al. eRNAs are required for p53-dependent enhancer activity and gene transcription. Molecular cell. 2013;49(3):524-35.

66. Lam MT, Cho H, Lesch HP, Gosselin D, Heinz S, Tanaka-Oishi Y, et al. Rev-Erbs repress macrophage gene expression by inhibiting enhancer-directed transcription. Nature. 2013;498(7455):511-5.

67. Li W, Notani D, Ma Q, Tanasa B, Nunez E, Chen AY, et al. Functional roles of enhancer RNAs for oestrogen-dependent transcriptional activation. Nature. 2013;498(7455):516-20.

68. Sigova AA, Mullen AC, Molinie B, Gupta S, Orlando DA, Guenther MG, et al. Divergent transcription of long noncoding RNA/mRNA gene pairs in embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(8):2876-81.

69. Feng J, Bi C, Clark BS, Mady R, Shah P, Kohtz JD. The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator. Genes Dev. 2006;20(11):1470-84.

70. Wang KC, Chang HY. Molecular Mechanisms of Long Noncoding RNAs. Molecular cell. 2011;43(6):904-14.

71. Kohtz JD, Fishell G. Developmental regulation of EVF-1, a novel non-coding RNA transcribed upstream of the mouse Dlx6 gene. Gene Expr Patterns. 2004;4(4):407-12.

72. Ng SY, Bogu GK, Soh BS, Stanton LW. The Long Noncoding RNA RMST Interacts with SOX2 to Regulate Neurogenesis. Molecular cell. 2013;51(3):349-59.

73. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell. 2007;129(7):1311-23.

74. Wang KC, Yang YW, Liu B, Sanyal A, Corces-Zimmerman R, Chen Y, et al. A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. Nature. 2011;472(7341):120-4.

75. Plath K, Mlynarczyk-Evans S, Nusinow DA, Panning B. Xist RNA and the mechanism of X chromosome inactivation. Annual review of genetics. 2002;36:233-78.

76. Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, et al. Long noncoding RNA as modular scaffold of histone modification complexes. Science (New York, NY). 2010;329(5992):689-93.

77. Caley DP, Pink RC, Trujillano D, Carter DR. Long noncoding RNAs, chromatin, and development. ScientificWorldJournal. 2010;10:90-102.

78. Mercer TR, Mattick JS. Structure and function of long noncoding RNAs in epigenetic regulation. Nature structural & molecular biology. 2013;20(3):300-7.

79. Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. Annu Rev Biochem. 2012;81:145-66.

80. Kim HD, Choe HK, Chung S, Kim M, Seong JY, Son GH, et al. Class-C SOX transcription factors control GnRH gene expression via the intronic transcriptional enhancer. Mol Endocrinol. 2011;25(7):1184-96.

81. Kino T, Hurt DE, Ichijo T, Nader N, Chrousos GP. Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. Science signaling. 2010;3(107):ra8.

82. Kagey MH, Newman JJ, Bilodeau S, Zhan Y, Orlando DA, van Berkum NL, et al. Mediator and cohesin connect gene expression and chromatin architecture. Nature. 2010;467(7314):430-5.

83. Phillips-Cremins JE, Sauria ME, Sanyal A, Gerasimova TI, Lajoie BR, Bell JS, et al. Architectural protein subclasses shape 3D organization of genomes during lineage commitment. Cell. 2013;153(6):1281-95.

84. Lai F, Orom UA, Cesaroni M, Beringer M, Taatjes DJ, Blobel GA, et al. Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. Nature. 2013;494(7438):497-501.

85. Korostowski L, Sedlak N, Engel N. The Kcnq1ot1 long non-coding RNA affects chromatin conformation and expression of Kcnq1, but does not regulate its imprinting in the developing heart. PLoS genetics. 2012;8(9):e1002956.

86. Clemson CM, Hutchinson JN, Sara SA, Ensminger AW, Fox AH, Chess A, et al. An architectural role for a nuclear noncoding RNA: NEAT1 RNA is essential for the structure of paraspeckles. Molecular cell. 2009;33(6):717-26.

87. Tian D, Sun S, Lee JT. The long noncoding RNA, Jpx, is a molecular switch for X chromosome inactivation. Cell. 2010;143(3):390-403.

88. Engreitz JM, Pandya-Jones A, McDonel P, Shishkin A, Sirokman K, Surka C, et al. The Xist lncRNA exploits three-dimensional genome architecture to spread across the X chromosome. Science (New York, NY). 2013;341(6147):1237973.

89. Beltran M, Puig I, Pena C, Garcia JM, Alvarez AB, Pena R, et al. A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition. Genes Dev. 2008;22(6):756-69.

90. Zong X, Tripathi V, Prasanth KV. RNA splicing control: yet another gene regulatory role for long nuclear noncoding RNAs. RNA Biol. 2011;8(6):968-77.

91. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell. 2011;147(2):358-69.

92. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, et al. The transcriptional landscape of the mammalian genome. Science (New York, NY). 2005;309(5740):1559-63.

93. Hangauer MJ, Vaughn IW, McManus MT. Pervasive Transcription of the Human Genome Produces Thousands of Previously Unidentified Long Intergenic Noncoding RNAs. PLoS Genet. 2013;9(6):e1003569.

94. Wetsel WC, Valenca MM, Merchenthaler I, Liposits Z, Lopez FJ, Weiner RI, et al. Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. Proc Natl Acad Sci U S A. 1992;89(9):4149-53.

95. Wetsel WC, Mellon PL, Weiner RI, Negro-Vilar A. Metabolism of pro-luteinizing hormone-releasing hormone in immortalized hypothalamic neurons. Endocrinology. 1991;129(3):1584-95.

96. Weiner RI, Martinez de la Escalera G. Pulsatile release of gonadotrophin releasing hormone (GnRH) is an intrinsic property of GT1 GnRH neuronal cell lines. Hum Reprod. 1993;2(13):13-7.

97. Maggi R, Pimpinelli F, Molteni L, Milani M, Martini L, Piva F. Immortalized luteinizing hormone-releasing hormone neurons show a different migratory activity in vitro. Endocrinology. 2000;141(6):2105-12.

98. Nelson SB, Lawson MA, Kelley CG, Mellon PL. Neuron-specific expression of the rat gonadotropin-releasing hormone gene is conferred by interactions of a defined promoter element with the enhancer in GT1-7 cells. Molecular endocrinology (Baltimore, Md. 2000;14(9):1509-22.

99. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature. 2009;458(7235):223-7.

100. Orom UA, Shiekhattar R. Long non-coding RNAs and enhancers. Current opinion in genetics & development. 2011;21(2):194-8.

101. Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, et al. Galaxy: a platform for interactive large-scale genome analysis. Genome research. 2005;15(10):1451-5.

102. Blankenberg D, Gordon A, Von Kuster G, Coraor N, Taylor J, Nekrutenko A. Manipulation of FASTQ data with Galaxy. Bioinformatics (Oxford, England). 2010;26(14):1783-5.

103. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome biology. 2013;14(4):R36.

104. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature biotechnology. 2010;28(5):511-5.

105. Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat Biotech. 2011;29(1):24-6.

106. Thorvaldsdóttir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Briefings in Bioinformatics. 2013;14(2):178-92.

107. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: A laboratory manual. New York: Cold Spring Harbor Laboratory; 1982.

108. Poling MC, Kim J, Dhamija S, Kauffman AS. Development, sex steroid regulation, and phenotypic characterization of RFamide-related peptide (Rfrp) gene expression and RFamide receptors in the mouse hypothalamus. Endocrinology. 2012;153(4):1827-40.

109. Simmons DG, Kennedy TG. Induction of glucose-regulated protein 78 in rat uterine glandular epithelium during uterine sensitization for the decidual cell reaction. Biol Reprod. 2000;62(5):1168-76.

110. Givens ML, Kurotani R, Rave-Harel N, Miller NL, Mellon PL. Phylogenetic footprinting reveals evolutionarily conserved regions of the gonadotropin-releasing hormone gene that enhance cell-specific expression. Mol Endocrinol. 2004;18(12):2950-66.

111. Larder R, Mellon PL. Otx2 induction of the gonadotropin-releasing hormone promoter is modulated by direct interactions with Grg co-repressors. The Journal of biological chemistry. 2009;284(25):16966-78.

112. Givens ML, Kurotani R, N. R-H, Miller NLG, Mellon PL. Phylogenetic footprinting reveals functional upstream regions of the gonadotropin-releasing hormone gene that enhance cell-specific expression. Molecular endocrinology (Baltimore, Md. 2004;18:2950-66.

113. Brayman MJ, Pepa PA, Mellon PL. Androgen receptor repression of gonadotropin-releasing hormone gene transcription via enhancer 1. Mol Cell Endocrinol. 2012;363(1-2):92-9.

114. Kong L, Zhang Y, Ye ZQ, Liu XQ, Zhao SQ, Wei L, et al. CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. Nucleic acids research. 2007;35(Web Server issue):W345-9.

115. De Santa F, Barozzi I, Mietton F, Ghisletti S, Polletti S, Tusi BK, et al. A large fraction of extragenic RNA pol II transcription sites overlap enhancers. PLoS Biol. 2010;8(5):e1000384.

116. Larder R, Mellon PL. Otx2 induction of the gonadotropin-releasing hormone promoter is modulated by direct interactions with Grg co-repressors. J Biol Chem. 2009;284(25):16966-78.

117. Wang D, Garcia-Bassets I, Benner C, Li W, Su X, Zhou Y, et al. Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA. Nature. 2011;474(7351):390-4.

118. Maamar H, Cabili MN, Rinn J, Raj A. linc-HOXA1 is a noncoding RNA that represses Hoxa1 transcription in cis. Genes Dev. 2013;27(11):1260-71.

119. Sun M, Kraus WL. From discovery to function: the expanding roles of long noncoding RNAs in physiology and disease. Endocrine reviews. 2015;36(1):25-64.

120. Gupta R, Ezashi T, Roberts RM. Squelching of ETS2 transactivation by POU5F1 silences the human chorionic gonadotropin CGA subunit gene in human choriocarcinoma and embryonic stem cells. Mol Endocrinol. 2012;26(5):859-72.

121. Gill G, Ptashne M. Negative effect of the transcriptional activator GAL4. Nature. 1988;334:721-4.

122. Chu C, Qu K, Zhong FL, Artandi SE, Chang HY. Genomic Maps of Long Noncoding RNA Occupancy Reveal Principles of RNA-Chromatin Interactions. Molecular cell. 2011.

123. Huarte M, Guttman M, Feldser D, Garber M, Koziol MJ, Kenzelmann-Broz D, et al. A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. Cell. 2010;142(3):409-19.

124. Novaira HJ, Sonko ML, Radovick S. Kisspeptin induces dynamic chromatin modifications to control GnRH gene expression. Mol Neurobiol. 2015;in press.

125. Novaira HJ, Fadoju D, Diaczok D, Radovick S. Genetic mechanisms mediating kisspeptin regulation of GnRH gene expression. J Neurosci. 2012;32(48):17391-400.

126. Pandey RR, Mondal T, Mohammad F, Enroth S, Redrup L, Komorowski J, et al. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. Molecular cell. 2008;32(2):232-46.

127. Novaira HJ, Yates M, Diaczok D, Kim H, Wolfe A, Radovick S. The gonadotropin-releasing hormone cell-specific element is required for normal puberty and estrous cyclicity. J Neurosci. 2011;31(9):3336-43.