UNIVERSITY OF CALIFORNIA, SAN DIEGO

Transcriptional Control of the Gonadotropin-Releasing Hormone Gene in Development

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

Doctor of Philosophy

in
Biomedical Sciences

by

Nichol L. G. Miller

Committee in charge:

Professor Pamela L. Mellon, Chair
Professor Joseph G. Gleeson
Professor Mark A. Lawson
Professor William McGinnis
Professor Nicholas J.G. Webster

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This Dissertation of Nichol L. G. Miller is approved, and it is acceptable in quality and form for publication in microfilm:

Chair

University of California, San Diego

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

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Additional portions of the material contained in Chapter II are, in part, a manuscript in preparation entitled "Evolutionarily Conserved Regions of the

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VITA

1996-2000	B.S., Biological Science, University of California, Santa Barbara
2000-2001	Research Assistant, University of California, Santa Barbara
2008	Ph.D., Biomedical Sciences, University of California, San Diego

AWARDS AND HONORS

2002-2003	NIH Training Grant, Neuroplasticity of Aging, University of California, San Diego
2002-2005	NIH Training Grant, Genetics, University of California, San Diego
2007	The Endocrine Society Travel Grant Award

PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

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Nichol L. G. Miller

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Professor Pamela L. Mellon, Chair

Reproductive endocrinology is focused on the hypothalamic-pituitary-gonadal (HPG) axis. The gonadotropin-releasing hormone (GnRH) neurons of the hypothalamus are the primary regulators of the HPG axis and thus, reproductive function in mammals. Therefore, the molecular and developmental properties of GnRH neurons are important for our knowledge of reproductive function and our understanding of infertility. GnRH gene expression is restricted to a dispersed population of neurons within the hypothalamus. These studies elucidate some of the molecular processes that contribute to this strict specification. The research presented in this dissertation utilizes both immortalized cell culture and genetically modified animal models to study the regulation of the GnRH gene and neuron. Herein are described the identification of novel distal *cis*-regulatory elements and

trans-regulatory proteins that contribute to GnRH gene expression and neuronal specification during development.

While previous research identified and characterized some *cis*-regulatory elements, recent evidence suggested that additional upstream sequences might provide further specification for proper targeting to the GnRH neurons. Studies characterizing the upstream enhancer within the rat GnRH regulatory sequence revealed Nuclear Factor 1 as a new regulator of the GnRH gene. Using DNA sequence from the recently completed rat genome and an *in silico* approach termed phylogenetic footprinting, two novel, evolutionarily conserved elements were identified and shown to regulate neuron-specific gene expression of GnRH.

Prader-Willi syndrome (PWS) is a human contiguous multigene disorder characterized by obesity, hypothalamic hypogonadism, and developmental delay. The genetic cause of PWS is the loss of expression from the 15q11-13 chromosomal region. This region contains several genes, including one that encodes the MAGE family member, necdin. The research presented herein demonstrates the necessity of necdin for proper GnRH gene expression and neuronal development. The findings provide a mechanism by which the loss of necdin could contribute to the infertility observed in PWS.

This dissertation utilizes both *in vitro* and *in vivo* approaches to elucidate important regulators of GnRH gene transcription and neuronal migration in development and significantly contributes to our understanding of reproductive control by the hypothalamic GnRH neurons.

CHAPTER I

INTRODUCTION

The Hypothalamic-Pituitary-Gonadal Axis

Mammalian reproductive function is dependent on endocrine signaling within the hypothalamic-pituitary-gonadal (HPG) axis. Hormones in the HPG axis are responsible for gonadal function and thus, reproduction. Neuroendocrine signals from the hypothalamus are at the apex of the HPG axis. While several populations of cells in the central nervous system (CNS) are known to affect neuroendocrine signaling, the gonadotropin-releasing hormone (GnRH) neurons are absolutely necessary for fertility. GnRH neurons are unique in that they are scattered throughout the septohypothalamic region (Schwanzel-Fukuda et al., 1992; Schwanzel-Fukuda and Pfaff, 1989) and they specifically synthesize the GnRH decapeptide. GnRH neurons project axons into the median eminence where GnRH is released in a pulsatile manner directly into the hypophyseal portal blood system. This carries secreted GnRH to the anterior pituitary where it binds its receptor (GnRH-R) on gonadotrope cells and differentially regulates the expression and pulsatile secretion of the gonadotropin hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These gonadotropin hormones are released into the bloodstream and stimulate LH and FSH

receptors on cells the gonads to regulate oogenesis and ovulation in females and spermatogenesis in males. The gonads also release steroid hormones that inhibit secretion of GnRH from the hypothalamus and both negatively and positively affect gonadotropin hormones from the anterior pituitary (Shupnik, 1996). Proper regulation of all of these hormones is critical for maintaining fertility.

The GnRH Neuron

As the critical cell at the apex of the HPG axis, and therefore reproduction, the GnRH neuron is an important area of reproductive research. In mice, the GnRH gene is expressed in a spatiotemporal manner during embryonic development. GnRH neurons develop in a unique fashion as they are first detected outside the central nervous system. They originate in the vomeronasal organ and medial basal wall of the olfactory placode at approximately embryonic day 10.5 (e10.5) (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989b) and begin expressing GnRH mRNA by e11.5 (Wray et al., 1989b). By e12.5, most of the full population of GnRH neurons are established and the GnRH peptide is detectable (Wray et al., 1989b). Coincident with development these neurons migrate across the nasal septum and into the forebrain. GnRH neuronal migratory pathways are closely associated with the peripherin-positive olfactory neuron axons (Wray et al., 1994). These olfactory neurons migrate from the olfactory pit into the olfactory bulb prior to the migration of the GnRH neurons and are hypothesized to guide the GnRH neurons along their proper migratory

route from the nose into the CNS, where most reach their place in the hypothalamus by e16.5. Only then can GnRH neurons send out axons and execute their role in the HPG axis, as neurosecretory cells. In this way, GnRH neuron migration, development, and expression of GnRH all occur coincidentally and may, in fact, be mechanistically related.

GnRH Neuronal Models

Due to the dispersed nature of the scarce GnRH neuronal population, it has been difficult to study their molecular mechanisms *in vivo*. To allow for molecular studies of transcription, translation, and secretion of GnRH, targeted oncogenesis was used to create immortalized, differentiated cell lines that express GnRH (Mellon et al., 1990; Radovick et al., 1991). Three kb of the 5' regulatory region of the rat GnRH gene was fused to the coding region of the SV40 T antigen to create a tumor in the forebrain. The GT1 cell culture was derived from this tumor and subcloned into three cell lines: GT1-1, GT1-3, and GT1-7. These cell lines have characteristics consistent with hypothalamic neurosecretory GnRH neurons (Mellon et al., 1990) such as projecting neurites, secreting GnRH in response to depolarization, and expressing several neuronal markers (Favit et al., 1993; Wetsel et al., 1993). Additionally, GT1-1 and GT1-7 cells have intrinsic pulsatile release of GnRH (Weiner and Martinez de la Escalera, 1993; Wetsel et al., 1992). GT1-7 cells have been shown to functionally substitute for GnRH neurons when implanted into the mouse third ventricle with a

hypogonadal background (Silverman et al., 1992). In addition, the GT1-7 cells have been used extensively to study the effect of neurotransmitters, growth factors, steroid hormones, and many other factors on the regulation of GnRH gene expression. The above data suggest that the GT1-7 line represents a mature and differentiated GnRH-producing cell model system.

The Gn11 and NLT cell lines were created using a similar approach (Radovick et al., 1991). One kb of the human GnRH regulatory region was used to drive SV40 T antigen, forming a tumor in the olfactory region of the mouse. The Gn11 and NLT cell lines were derived from one tumor and were characterized for their ability to migrate in culture, but both only release a very small amount of GnRH peptide, suggesting they represent immature GnRH neurons. Both the GT1-7 and NLT/Gn11 model GnRH cell lines have been important tools for studying the development of the GnRH neurons at molecular level.

Regulation of GnRH

The limited expression of GnRH to a scattered population of neurons is dependent on cis-regulatory elements. However, not all elements governing GnRH gene expression have been elucidated. The GT1-7 immortalized cell line has been used in our laboratory and in others to analyze the molecular control of GnRH peptide and the development of the neurons themselves. Previous studies indicate that specific expression of GnRH is controlled at the transcriptional level (Kepa et al., 1996;

Lawson et al., 2002; Pape et al., 1999; Skynner et al., 1999b; Suter et al., 2000; Whyte et al., 1995). To date, three discrete regulatory elements have been identified and characterized in the extensively studied rat GnRH gene. A 173 bp promoter, just upstream of the start site (Eraly and Mellon, 1995), a 300 bp enhancer at approximately -1863 to -1571 relative to the start site (Whyte et al., 1995), and an additional 350 bp upstream enhancer at -2980 to -2631 (Givens et al., 2004). Interestingly, all three of these regulatory regions are evolutionarily conserved across human, rat, and mouse species and also contain conserved blocks of transcription factor binding sites that have been shown to regulate the GnRH gene.

Cell-restricted expression of GnRH has been analyzed using transgenic mouse models. A transgenic mouse with the rat GnRH 173 bp promoter and the 300 bp enhancer driving the LacZ gene was capable of specifically targeting expression to GnRH neurons, but not all neurons were targeted, (approximately 50-60%) (Lawson et al., 2002). Additionally, a transgene using 3.8 kb upstream of the human GnRH gene targets luciferase activity to the hypothalamus, however, cell-specific targeting accuracy was not specifically addressed (Wolfe et al., 1996). Transgenic mice containing 5.2 and 3.4 kb of the mouse upstream regions (Pape et al., 1999; Skynner et al., 1999b; Suter et al., 2000) target most of the GnRH neuron population. Conversely, a transgene using 2.1 or 1.7 kb of the mouse sequence targeted few or no GnRH neurons, respectively (Pape et al., 1999). Therefore, it is likely that additional regulatory regions are necessary to target specific expression of the GnRH gene.

Also contributing to the knowledge base of GnRH gene regulation is the identification of several transcriptional regulators that significantly induce or repress expression by binding to the characterized regulatory regions. These include Oct-1 (Clark and Mellon, 1995), GATA-4 (Lawson et al., 1996), Pbx/Prep (Rave-Harel et al., 2004), Otx2 (Kelley et al., 2000), Msx and Dlx (Givens et al., 2005), among others. Figure 1-1 depicts a more comprehensive view of transcription factor regulation of GnRH. Additionally, we have recently shown that Grg proteins serve as co-factors for the Oct-1 and Msx1 transcription factors and have been shown to play a role in GnRH gene regulation (Rave-Harel et al., 2005). Therefore, it is probable that specific combinations of co-factors contribute specificity to the transcriptional regulators. However, none of these factors are unique to GnRH neurons and most utilize DNA-binding sites with broad specificity. Thus, the mechanism by which these factors specify GnRH gene expression to only 800 neurons during their development and migration, is as yet unknown.

GnRH mRNA and promoter activity were also shown to vary according to location and developmental stage of the GnRH neuron (Simonian and Herbison, 2001). GnRH promoter activity is very low in the GnRH neurons within the nasal regions, however, activity increases as the neurons migrate toward the cribriform plate, and is high in the GnRH neurons in the forebrain. Therefore, it appears that the activity of the GnRH promoter is also dependent upon the location or maturation of the neuron, adding additional levels of complexity to the study of the expression of the GnRH gene.

It is also of importance to note that several factors have been identified as specifically regulating the migration of GnRH neurons. Functionality of these neuroendocrine cells relies not only on their synthesis and secretion of the GnRH peptide, but also on proper migration. These factors include small secreted signaling molecules [gamma-aminobutyric acid (GABA) (Bless et al., 2005; Bless et al., 2000), cholocystokinin (Giacobini et al., 2004), adhesion molecules such as polysialylated neural adhesion molecule (PSA-NCAM) (Yoshida et al., 1999), growth factors and their receptors (fibroblast growth factor (Tsai et al., 2005) and hepatocyte growth factor (Giacobini et al., 2007), and soluble factors and their receptors (Netrin 1(Schwarting et al., 2004), stromal cell-derived factor-1 (Schwarting et al., 2006), Gas6 (Nielsen-Preiss et al., 2007), and others such as nasal embryonic luteinizing hormone-releasing hormone factor (NELF) (Kramer and Wray, 2000). Interestingly, yet consistent with the regulation of GnRH gene expression, these factors regulating migration are not specific to GnRH neurons and seem to work on a heterogeneous population of GnRH neurons (Tobet et al., 1996; Todman et al., 2005)]. In addition, recent evidence suggests that there might also be a mechanical component to GnRH neuronal migration (Bless et al., 2005). Kisspeptin and its receptor GPR54 also play a role in GnRH neuron activation in puberty (d'Anglemont de Tassigny et al., 2007).

Importance of GnRH in Reproductive Health Research

As GnRH is the central regulator of the HPG axis, it is at the top of the cascade of events which are ultimately responsible for reproductive function. That the GnRH gene is necessary for fertility in mammals is shown by a natural mutation in the hypogonadal mouse that deletes most of the GnRH coding sequence (Mason et al., 1986) and verified by human studies of hypogonadotrophic hypogonadism and Kallmann syndrome (Seminara et al., 1998). Without normal GnRH expression and secretion, puberty will not occur, even if downstream components of the HPG axis are normal. It is of interest to note that although most research has been done on GnRH (also termed GnRH-I), GnRH-II, expressed in some mammals, and GnRH-III, found in fish, have also been recently characterized. However, these genes are not found in mice where mutation of the GnRH-I gene causes complete infertility, and therefore, are not being addressed in this disseration.

GnRH deficiency is associated with several genetic disorders. Kallmann syndrome has been the most extensively studied. It is heterogeneous at both the clinical and genetic level. Clinical presentation includes hypogonadotrophic hypogonadism and congenital anosmia or hyposmia. Genetic causes identified to date include KAL1 (X-linked) (Franco et al., 1991; Legouis et al., 1991), fibroblast growth factor receptor 1 (FGFR1) (autosomal dominant) (Dode et al., 2003; Pitteloud et al., 2006a; Pitteloud et al., 2006b; Tsai and Gill, 2006), prokineticin 2 (PROK2) and its receptor (PROKR2) (Dode et al., 2006). Additionally, cases lacking olfactory defects

have attributed to mutations in GnRH-R (Beranova et al., 2001; de Roux et al., 1997; Seminara et al., 2000), GPR54 (de Roux et al., 2003), and FGFR1 (Trarbach et al., 2007). Other idiopathic hypogonadotrophic hypogonadism (IHH) have been identified in adrenal hypoplasia congenital (AHC) due to a mutation in the NROB1 gene (Habiby et al., 1996), and in leptin deficiency (Strobel et al., 1998). Mutations in several genes affecting pituitary gonadotropins also result in IHH (Layman, 2003). Other genetic syndromes resulting in hypogonadism include chromosome disorders such as Turner and Kleinfelter syndromes and the contiguous gene disorder, Prader-Willi syndrome (PWS). The source of the infertility in Prader-Willi syndrome has not yet been explained.

Ultimately, amenorrhea (Reame et al., 1985), precocious puberty (Strauss and Barbieri, 2004), and infertility can result if GnRH neurons are not properly regulated (Marshall et al., 1991). Also, pulsatility of GnRH can be affected by other factors such as anorexia nervosa, excessive exercise, stress, and malnutrition (Eagleson et al., 2000; Lado-Abeal et al., 1998; Pastor et al., 1998; Rivier and Rivest, 1991). This is especially true in the female where precise hormonal balances govern so much of reproductive physiology. Important discoveries in contraception and fertility are dependent upon our knowledge of reproductive physiology, which is becoming increasingly understood because of advances in the molecular biology of reproduction.

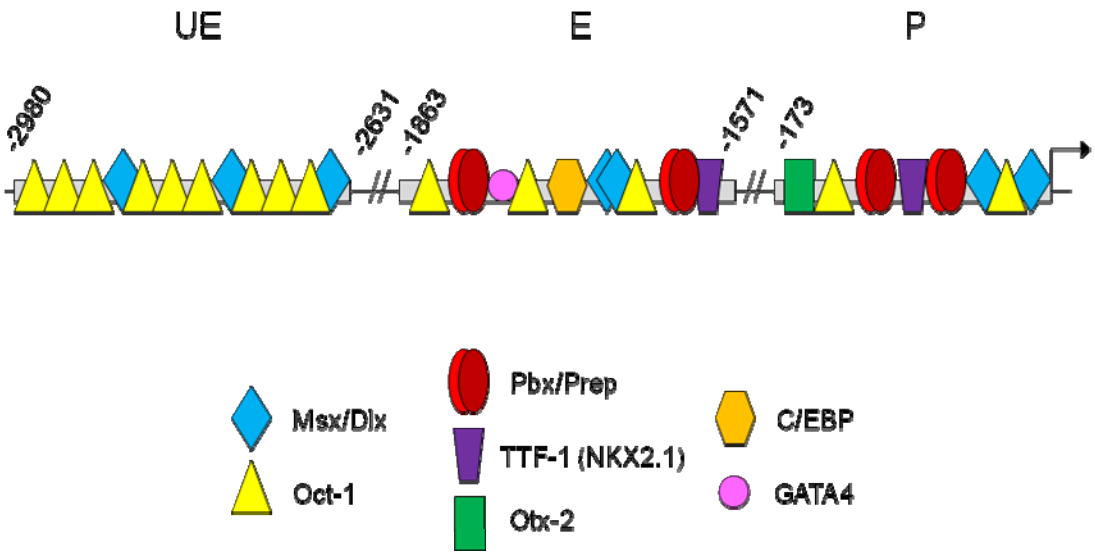

Figure 1-1. Transcriptional Regulators of GnRH. The diagram shows the characterized regulatory elements of the rat GnRH gene. UE – upstream enhancer, E – enhancer, P – promoter. Previous studies have identified several transcription factors that bind to these regulatory regions, depicted as colored shapes.

CHAPTER II

Evolutionarily Conserved Regions of the Gonadotropin-releasing Hormone Gene Enhance Neuronal Specification to Differentiated Neurons

Gonadotropin-releasing hormone (GnRH) is the central regulator of the hypothalamic-pituitary-gonadal (HPG) axis, and thus is critical for reproductive function. GnRH gene expression is restricted to a small, dispersed population of neurons in the hypothalamus. The mechanisms by which GnRH expression is restricted to this specific subset of neurons are not fully understood. Proper targeting to these neurons requires upstream regulatory elements that have not been completely characterized. Thus far, three regions have been identified and characterized in the rat gene, the 173 bp promoter (-173 to +1), a 292 bp enhancer (-1863 to -1572), and the 349 bp upstream enhancer (-2980 to -2631). All three are evolutionarily conserved across species and confer neuronal specificity *in vitro* in the GT1-7 GnRH neuronal cell line. Additionally, the enhancer and promoter specifically target GnRH neurons *in vivo*, but fail to target the entire population. These three characterized regulatory elements also contain binding sites for transcription factors that are necessary for GnRH transcriptional regulation. In the present study, we identify a novel transcriptional regulator of GnRH, Nuclear Factor 1 (NF1), which functions cooperatively with Oct-1. We also perform phylogenetic footprinting sequence

comparison between human and rodent upstream GnRH regions to identify four novel regions between -3 kb and -5 kb (regions 1-4). The homology in these elements was confirmed using two *in silico* techniques, pairwise BLAST analysis and VISTA plot comparison. The importance of this far-upstream sequence was established by -5 kb rat GnRH reporter activity that exceeded the activity of the previously characterized elements. Truncation analysis of the -5 kb rat GnRH sequence identified two of the novel regions, 1 and 3, as critical for GnRH promoter activity and confirmed the necessity of the characterized promoter, enhancer, and upstream enhancer. In fact, region 1 appears to be a continuation of the upstream enhancer that was not identified in prior studies due to the incomplete rat genome sequence available at that time. Not only are these novel regions evolutionarily conserved, but they also contain putative binding sites for elements known to regulate GnRH both *in vitro* and *in vivo*. Thus, experimental and *in silico* data show that additional upstream cis-regulatory regions are important for GnRH gene regulation.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH), a hypothalamic neuropeptide, is the central regulator of reproductive function, acting through the hypothalamic-pituitary-gonadal (HPG) axis. GnRH gene expression is restricted to specific GnRH neurons and the peptide is secreted in a pulsatile manner to control reproduction. GnRH neurons [~800 in the mouse, (Wray et al., 1989a)] are dispersed throughout the hypothalamus. Research in the field indicates that this limited and specific expression of GnRH is controlled at the level of transcription (Kepa et al., 1996; Lawson et al., 2002; Pape et al., 1999; Skynner et al., 1999b; Suter et al., 2000; Whyte et al., 1995).

To date, three discrete regulatory elements have been identified and characterized in the extensively studied rat GnRH gene. A 173 bp promoter, just upstream of the start site (Eraly and Mellon, 1995), a 292 bp enhancer at approximately -1863 to -1571 relative to the start site (Whyte et al., 1995), and an additional 349 bp upstream enhancer at -2980 to -2631 (Givens et al., 2004). All three of these regulatory regions are evolutionarily conserved across human, rat, and mouse species and also contain conserved blocks of transcription factor binding sites that have been shown to regulate the GnRH gene (Givens et al., 2004). This *in silico* evidence suggests that these regions may play a significant role in GnRH regulation. One test of whether we have identified all the regions responsible for transcriptional control of GnRH would be whether the identified regulatory regions responsible for GnRH regulation can specifically and correctly target 100% of GnRH neurons *in vivo*.

Thus far, this has not been accomplished. A transgenic mouse with the rat GnRH 173 bp proximal promoter and the 292 bp enhancer driving the β-galactosidase reporter gene was capable of specifically targeting expression to GnRH neurons, but the complete population was not targeted (approximately 50-60%) (Lawson et al., 2002). The full 3 kb upstream of the GnRH start site targets additional GnRH neurons but also expresses the transgene in non-specific regions of the hypothalamus. Additionally, transgenic mice using human (Wolfe et al., 1996) and mouse (Pape et al., 1999; Skynner et al., 1999b; Suter et al., 2000) upstream regions have been created, but neither has been shown to target all of the GnRH neurons *in vivo* and both show significant ectopic overexpression. Specifically, work done *in vivo* with the mouse GnRH gene suggests that elements 5' to the characterized regions could increase the number of GnRH neurons targeted (Pape et al., 1999). In fact, there seems to be a significant drop in the number of neurons targeted from -5.2 or -3.4 to -2.1 kb. This suggesting that there are additional regulatory regions necessary to specifically target GnRH neurons, in addition to the characterized enhancer and promoter and they may lie upstream of these regions.

To search for these regions, the method of phylogenetic footprinting (comparative analysis of genomes across species) was utilized. Previously, our lab used this technique to identify the upstream enhancer and confirm the proximal promoter and enhancer (Givens et al., 2004). The functional importance of the proximal promoter and enhancer were confirmed *in vitro* (Whyte et al., 1995) and *in vivo* (Lawson et al., 2002). Additionally, the upstream enhancer was identified and

characterized *in vitro* and significantly and specifically regulates GnRH gene expression in GT1-7 cells. However, this regulatory region has not been tested for its targeting efficiency *in vivo*. As was suggested by Pape, et al. (Pape et al., 1999), there could be additional regulatory regions upstream of the three characterized elements and phylogenetic footprinting could be used to help identify them.

In addition, multiple molecular studies in our lab and others have confirmed the importance of these regions, the binding sites they contain, and their respective transcription factors in the GT1-7 cells *in vitro* (Mellon et al., 1990). These include Oct-1 (Clark and Mellon, 1995), GATA (Lawson et al., 1996), Pbx/Prep (Rave-Harel et al., 2004), Otx-2 (Kelley et al., 2000), Msx and Dlx (Givens et al., 2005) among others. However, none of these factors are specific to GnRH neurons and most of these transcription factors are homeodomain proteins that can bind to low affinity sites within DNA. Thus, the challenge is to elucidate how ubiquitous and promiscuous transcription factors, such as homeodomain proteins, can regulate GnRH in a manner specific enough to control the neurons' GnRH gene expression, development, and migration, which is all occurring concurrently. We have recently shown that Grg proteins serve as co-factors for the Oct-1 and Msx-1 transcription factors and have been shown to play a role in GnRH gene regulation (Rave-Harel et al., 2005). It is probable that specific combinations of co-factors contribute specificity to the transcriptional regulators.

As GnRH is the essential regulator of the hypogonadal-pituitary axis, it is at the top of the cascade of events that are ultimately responsible for reproductive

function. To fully understand how the reproductive system operates, at a molecular level in the hypothalamus, it is important to learn as much as possible about how GnRH is regulated. Without normal GnRH expression and secretion, reproduction will not occur properly, even if downstream components of the HPG axis are normal. We have shown that a novel factor, Nuclear Factor 1 (NF1), binds the previously characterized upstream enhancer, that multiple NF1 family members are expressed in GT1-7 cells, and that NF1 is capable of regulating GnRH gene expression through Oct-1. We have also identified additional distal GnRH regulatory elements that are evolutionarily conserved across species, contribute to neuron-specific regulation, and have several putative binding sites for transcription factors known to regulate GnRH. These results suggest important roles for these conserved DNA regions in the accurate expression of the GnRH gene.

RESULTS

Identification of a Novel Complex Binding in the Upstream Enhancer

We previously identified several sequence motifs in the GnRH upstream enhancer and promoter as binding Oct-1 (Givens et al., 2004). We performed electromobility shift assays (EMSA) using GT1-7 nuclear extract to confirm binding to these sites within the upstream enhancer. In addition to the protein complex corresponding to Oct-1 (Figure 2-1A, arrow 2), a second, faster mobility complex was observed (arrow 1). Additionally, a band that co-migrated with this protein complex was also identified on all nine of the probes from the upstream enhancer in addition to all Oct-1 binding sites in the enhancer and promoter (Givens et al., 2004). Oligonucleotide probes were generated with mutations in the sequence both within and surrounding the Oct-1 binding sequence. While mutations within the consensus Oct-1-binding sequence reduced the binding of the Oct-1 band (Figure 2-1A, lane 3), when sequence flanking the Oct-1 site was mutated, binding of the faster mobility complex was competed (Figure 2-1A, lane 4 mOCT)(Givens et al., 2004). Upon closer examination, we observed that this mutation changed the sequence from CGAATGCAA**AT**CAC to CGAATGCAA**GC**CAC, and inadvertently created a high affinity binding site (AGCCA) for Nuclear Factor 1 (NF1) (Jones et al., 1987). NF1 has been shown to bind to the palindromic sequence, TTGGC(N_5)GCCAA (Borgmeyer et al., 1984), although it also binds to half sites of this sequence (Jones et

al., 1987; O'Connor and Bernard, 1995; Santoro et al., 1988). Based on these data, and the fact that NF1 is known to bind on both sides of Oct-1 binding sites in other systems (O'Connor and Bernard, 1995), we tested whether NF1 was present this complex binding the GnRH regulatory region.

We performed EMSA analysis again, using the radiolabeled probe corresponding to the upstream enhancer and GT1-7 nuclear extract. Consistent with the results reported above, an antibody to Oct-1 supershifted the Oct-1 band and the slower migrating complexes but did not shift the faster complex (Figure 2-1B, lane 3). The faster migrating complex was self-competed and when an anti-NF1 antibody was added, the faster-migrating complex (Figure 2-1B, arrow 1) was super-shifted (arrowhead), indicated that this complex, does, indeed, contain NF1. Additionally, it was observed that when increasing amounts of GT1-7 nuclear protein were added to the EMSA complex, several slower mobility complexes would appear (Figure 2-1B, arrows 3 and 4) that were also shifted in the presence of an anti-Oct-1 antibody, however the anti-NF1 antibody did not affect the slower mobility complexes. None of these complexes were shifted by a rabbit IgG antibody used as a non-specific antibody control. These data indicate that NF1 can bind to the upstream enhancer, together with or independent of Oct-1 and may be part of the slower migrating complexes observed.

NF1 Family Members Expressed in GT1–7 Cells

The NF1 family is made up of four genes, NF1A, B, C, and X (Rupp et al., 1990; Santoro et al., 1988). However, several splice variants exist for each family member (Kruse and Sippel, 1994). Having discovered this transcription factor binding to the upstream enhancer, enhancer, and promoter, we examined the expression pattern of NF1A, B, C, and X by RT-PCR in the model GnRH cell lines: GT1–7, the more mature GnRH-producing cell line (Mellon et al., 1990) and Gn11 and NLT, two immature representatives of the GnRH neuron derived from the same tumor (Radovick et al., 1991). We also examined NF1 expression in the pituitary gonadotrope cell line, LßT2, as well as mouse fibroblasts (NIH3T3). Figure 2-2 shows that NF1 family members A, B, C, and X are expressed in the GT1–7, Gn11, and NLT cells, whereas NIH3T3 cells express all of the NF1 family members and LβT2 cells do not express NF1A and B. Therefore, both of the GnRH model cell lines express mRNA transcripts from all four genes of the NF1 family.

We next sought to confirm the expression of NF1 at the protein level. We performed western blot analysis in GT1-7 nuclear extract and nuclear extract that was overexpressing the each of the NF1 family members using a polyclonal antibody recognizing NF1 (Figure 2-3A). To confirm the overexpression, these extracts were also western blotted for HA, since all of the expression vectors had HA tags (Figure 2-3B). While all of the NF1 proteins seemed to overexpress in the nuclear extracts, the only NF1 band that was present endogenously in the GT1-7 cells co-migrated with the

NF1C band (arrow, Figure 2-3). Furthermore, EMSA with GT1-7 nuclear extracts over-expressing the NF1A, NF1B, NF1C, and NF1X show that only over-expression of the NF1C variant significantly increases binding of the protein complex identified as NF1, and is shifted by an NF1 antibody (Figure 2-3C). This evidence suggests that NF1C may be the functional NF1 family member within the GT1-7 cells.

Dominant-Negative NF1 Affects Oct-1-Dependent Activation

It has been shown that NF1 physically and functionally associates with the homeodomain protein, TTF1 (Bachurski et al., 2003). That study used a dominant-negative, chimeric protein (NF1A/EN) containing the N-terminal region of the murine NF1A protein, which is capable of protein dimerization and DNA binding, fused to the N-terminal region of the *Drosophila* Engrailed. Thus, dominant-negative NF1 can form nonfunctional dimeric complexes through interaction with the N-terminal region of the chimeric protein. We used this dominant-negative hybrid protein to assess the contribution of NF1 and to explore the effect on transactivation potential through interaction with the homeodomain protein Oct-1 in transient transfection of GT1–7 cells. For this purpose, we used a reporter vector containing a multimer of the enhancer Oct-1/NF1 binding site (–1805 to –1766) driving luciferase expression. This element was chosen because it has been shown to be essential for GnRH expression (Clark and Mellon, 1995) and is responsive to Oct-1-mediated activation whereas the entire GnRH regulatory sequence is not (Rave-Harel et al., 2004). Interestingly,

addition of increasing amounts of dominant-negative NF1 (dnNF1) repressed reporter activity (Figure 2-4), indicating that NF1 contributes to GnRH promoter activity. Overexpression of Oct-1 resulted in an increase in reporter activity, but this increase was diminished by dominant-negative NF1 (Bachurski et al., 2003). These results suggest that a functional interaction occurs between Oct-1 and NF1. Overall, these data show that NF1 functions with Oct-1 to act on the upstream enhancer that our laboratory previously demonstrated to be important for targeting GnRH gene expression (Givens et al., 2004).

Phylogenetic Footprinting Reveals Four Novel Regions of Conserved Sequence in the GnRH Regulatory Region

It has previously been shown that GnRH expression is regulated at the transcriptional level (Kepa et al., 1996; Lawson et al., 2002; Pape et al., 1999; Skynner et al., 1999a; Suter et al., 2000; Whyte et al., 1995). Therefore, it is important to understand all of the transcriptional elements that play a role in GnRH gene regulation. Three elements, the promoter, enhancer, and upstream enhancer, have been characterized previously by our laboratory (Eraly and Mellon, 1995; Givens et al., 2004; Whyte et al., 1995). Interestingly, all three can be identified by a sequence alignment analysis termed phylogenetic footprinting which compares genomes across sequences. The full rat genome sequence has become available since the identification of the characterized rat GnRH regulatory elements, allowing for further analysis using

phylogenetic footprinting. Approximately -5 kb rat GnRH DNA sequence [2 kb more than analyzed previously (Givens et al., 2004)] was used in a pairwise "BLAST 2 sequences" alignment with the corresponding mouse and human sequences. It is of importance to note that we restricted analysis to -5 kb upstream of the transcriptional start site due to limits defined by the next gene's 3' coding sequence. Six regions of significant homology emerged from the rat and human alignment (Figure 2-5A). The most proximal region corresponds to the well-characterized, highly conserved, 173 bp promoter (Eraly and Mellon, 1995). The second area of homology contains the functional enhancer, which our laboratory demonstrated regulates the GnRH gene (Lawson et al., 2002; Whyte et al., 1995). The third region includes the upstream enhancer, which was characterized by our laboratory to further confer specificity to GnRH gene expression (Givens et al., 2004). Interestingly, additional sequence directly 5' to the upstream enhancer was identified in our alignment, due to more available rat genomic sequence available (Figure 2-5A). Furthermore, three more novel areas of strong homology distal to the previously described regions were identified (Figure 2-5). Region 1, is 153 bp corresponding to -3134 to -2982 in the rat GnRH sequence, has 80% homology to the human sequence, and is directly adjacent to the characterized upstream enhancer. Region 2 is 31 bp from -3588 to -3560 relative to the start site and is 96% homologous to the human. Region 3 is 304 bp, corresponds to -4198 to -3895, and has 82% homology. Finally, region 4 lies from -4491 to -4323, containing 227 bp that is 85% identical to the human sequence.

To confirm these data, we utilized VISTA as an additional method for alignment between the rat, mouse and human GnRH 5' sequence (Frazer et al., 2004). Figure 2-6 shows rat and human corresponding to approximately -5 kb to the GnRH transcriptional start site. The VISTA plot comparison identified 5 out of 6 BLAST analysis regions as highly homologous. Region 2 was not identified in the VISTA analysis, presumably because it is very short, since it is nearly identical between rodent and human. Interestingly, these novel areas of homology all have equivalent, if not higher, levels of identity between rat and human than do the previously characterized elements. Furthermore, these regions are also conserved in the bovine and canine genomes, lending additional support to the possibility of putative important regulatory elements being present in these novel sequences.

To further support the phylogenetic footprinting approach, *in silico* analysis was performed to look for consensus transcription factor binding elements within the conserved novel regions. As shown for the three previously characterized regulatory elements (Givens et al., 2004), clusters of binding sites were also identified specifically within the homologous regions. Interestingly, all four novel conserved regions contain putative binding sites for elements known to bind the GnRH promoter and regulate its gene expression. This indicates that, not only is there evolutionary conservation of these regulatory domains, but that transcription factor binding sites are found in clusters in higher density in the conserved regions and contain sites for factors known to regulate GnRH. These data suggest that these conserved far

upstream regions may contain regulatory activity important for proper expression of the GnRH gene.

Far Upstream Sequence Contributes to GnRH Gene Expression

Prior to testing the functionality of these novel homologous regions individually in GnRH gene regulation, we chose to confirm that this additional 2 kb of sequence that contains these four regions contributes to GnRH transcriptional activity. To do this, -4,984 bp of DNA sequence upstream of the rat GnRH gene mRNA start site was inserted in a plasmid driving luciferase expression. Transient transfections were performed in two GnRH neuronal cell lines, the differentiated GT1-7 cells express high levels of GnRH (Mellon et al., 1990) and the immature Gn11 cells express very low levels of GnRH (Radovick et al., 1991), as well as NIH3T3 fibroblasts as a control. The activity of the reporter plasmid containing the -4984 bp (-5 kb) rat GnRH, was compared to vectors containing the previously characterized regulatory elements, the GnRH promoter (P), enhancer (E), and upstream enhancer (UE) (Figure 2-7). As previously shown, addition of the upstream enhancer to the enhancer and promoter significantly increased GnRH transcriptional activity (Figure 2-7) (Givens et al., 2004). Interestingly, in GT1-7 cells, the full length -5 kb rat GnRH-luciferase reporter had significant increase (~30%) over the rat GnRH-UE/E/P-luciferase. Additionally, this increase occurred in a cell-specific manner in the GT1-7 cells, as the -5 kb reporter actually had significantly lower expression in the Gn11 and

NIH3T3 cells. The ratio of expression in GT1-7 cells versus Gn11 increases from 1.4 to 6.7 and from 12.0 to 322.1 when comparing to NIH3T3 cells. The fact that the -5 kb GnRH sequence activates GnRH gene expression and does so in a cell-specific manner, suggests that there are functional GnRH regulatory elements within this uncharacterized distal 2 kb region.

To further analyze this novel 2 kb of GnRH regulatory sequence and identify critical regions, we performed truncation analysis on the -5 kb rat GnRH-luciferase plasmid (Figure 2-8). Reporter vectors were made that sequentially truncated GnRH regulatory sequence from the 5' end. The -4491 rat GnRH truncation eliminates the most distal sequence, but conserved region 4 and everything downstream are intact. A -4199 rat GnRH vector truncated region 4 and the non-homologous sequence between it and region 3. Truncation to -3620 bp removed region 3 and most of the non-conserved sequence just downstream. The -3175 plasmid eliminated region 2 and some flanking non-homologous areas. Truncating to -2438 bp removed region 1 and the directly adjacent upstream enhancer. The -2168 vector truncated the non-conserved sequence between the upstream enhancer and enhancer. Truncation to -1380 bp and -957 eliminated the enhancer and 3' non-homologous regions, respectively.

When transiently transfected into GT1-7 cells, truncating from the full-length -5 kb to -4.4 kb or -4.1 kb (including region 4) did not significantly reduce reporter activity (Figure 2-8). However, when region 3 was eliminated in the -3.6 kb vector, activity was significantly reduced approximately 50%. Loss of region 2 in the -3.1 kb

reporter, actually significantly increased gene expression. As expected, truncation of region 1 and the upstream enhancer resulted in a loss of reporter activity, as did elimination of the enhancer sequence. Further deletion of the non-homologous intervening sequence, not surprisingly, did not affect basal reporter gene expression. Additionally, a -520 bp truncation was created, but transcriptional activity was too low to detect and so was not reported here. These data suggest that region 3, region 1/UE, and the previously characterized enhancer are all critical for full basal expression of the GnRH gene.

Novel Far Upstream Regions Confer Specificity to GnRH Gene Expression

We then analyzed the novel, conserved regions 3 and 1, individually, and in combination with the other characterized GnRH regulatory elements. We first tested region 3 by placing it upstream of the characterized promoter alone in a reporter vector driving luciferase. This resulted in a significant increase of GnRH reporter activity (Figure 2-9). A significant increase in activity was also observed when region 3 was placed upstream of the enhancer and promoter together. However, when region 3 was tested upstream of the previously characterized upstream enhancer, enhancer, and promoter together, no significant increase was seen. Additionally, when region 3 was used in combination with the upstream enhancer and the promoter (without the proximal enhancer), there was also no change in reporter activity. So, while region 3 is capable of enhancing gene expression of the promoter (and even more dramatically

so in the presence of the enhancer), addition of the upstream enhancer results in no change, suggesting some redundancy between region 3 and the upstream enhancer.

Since conserved region 1 appeared to be an extension of the previously characterized upstream enhancer, we wanted to test if addition of region 1 altered the effects of the upstream enhancer alone. As shown previously, when inserted upstream of the characterized GnRH promoter, the upstream enhancer did not increase reporter activity (Givens et al., 2004) (Figure 2-10). However when the additional 153 bp 3' of the upstream enhancer (region 1) was added, significantly enhanced promoter activity was observed. Additionally, adding region 1 to the upstream enhancer in the presence of the proximal enhancer also increased reporter activity. These results suggest that region 1 is indeed an extension of the upstream enhancer.

Conserved Regions Contain Putative Binding Sites

As mentioned above, the conserved regions contained several sequences corresponding to consensus sites for transcription factors known to bind GnRH in previously characterized regulatory elements and consequently regulate GnRH gene expression. We next examined these sequences and their potential binding proteins more closely, in the context of the novel conserved GnRH regulatory regions. We generated radiolabeled electromobility shift assay (EMSA), or gel shift, oligonucleotide probes corresponding to DNA sequences within the four novel conserved regions, regions 1-4, as shown in Figure 2-11. When these probes were

used in EMSA analysis with GT1-7 nuclear extract to assay for binding to GnRH regulatory regions, we observed several specific bands corresponding to proteins binding to all four novel regions (Figure 2-12). Protein bands were considered specific if they were competed by 100x unlabled oligonucleotides probe. Proteins binding to the -4401, -4014, and -3001 probes, which correspond to regions 4 and 1, were subjected to further examination by EMSA supershift analysis. Protein complexes bound to probe -4401 were supershifted by both anti-Pbx and anti-Prep antibodies (Figure 2-13A), indicating that Pbx and Prep proteins are capable of binding within this sequence in region 4. Additionally, an anti-Oct-1 antibody supershifted a protein complex bound to the -4014 and -3001 probes, corresponding to regions 4 and 1, respectively (Figure 2-13B). This demonstrates that Oct-1 can bind to GnRH regulatory sequence in these areas.

DISCUSSION

Appropriate GnRH gene expression is critical for reproduction in higher vertebrates. While the upstream sequences of GnRH differ across species, it appears that evolutionarily conserved regions are important for GnRH gene expression (Eraly and Mellon, 1995; Givens et al., 2004; Kepa et al., 1996; Kim et al., 2002). These upstream regions have been demonstrated to be functional *in vivo* through the creation of transgenic mouse models using these regulatory sequences to drive reporter gene expression (Lawson et al., 2002). Unfortunately, the characterized enhancer and promoter in the rat GnRH sequence were not sufficient to target all of the GnRH neurons (Lawson et al., 2002), although they did target expression specifically to large subset of GnRH neurons. Larger regulatory regions from rat, mouse, or human targeted more GnRH neurons in transgenic mice, but did not necessarily do so in a specific manner (Pape et al., 1999; Skynner et al., 1999b; Suter et al., 2000; Wolfe et al., 1996). Experiments using the mouse upstream sequence suggested that important targeting regions may lie even further upstream of the three rat GnRH regulatory elements that have been characterized thus far (Pape et al., 1999), between -5.2 and -3.4 in the corresponding mouse sequence. The results presented herein provide evidence that additional distal conserved regions contribute to the specificity of GnRH gene expression and that protein transcription factors that bind them also play a role in regulating GnRH transcription.

Cross-species comparison of the DNA sequence upstream of the GnRH start site, using phylogenetic footprinting, had previously elucidated three characterized GnRH regulatory elements in the rat sequence (Eraly and Mellon, 1995; Givens et al., 2004). The upstream enhancer was shown to enhance promoter activity in a GnRH neuronal cell line and to contribute to restricted gene expression of GnRH (Givens et al., 2004). Furthermore, this upstream enhancer binds Octamer-binding transcription factor 1, or Oct-1, a POU homeodomain transcription factor that participates in basal and hormone-induced regulation of the GnRH gene (Belsham and Mellon, 2000; Chandran and DeFranco, 1999; Clark and Mellon, 1995; Wolfe et al., 2002). Interestingly, upon investigating the binding of Oct-1 to the upstream enhancer, we identified a regulatory factor novel to GnRH gene expression, Nuclear Factor 1 (NF1). Our studies indicate that NF1 binds cooperatively with Oct-1 to the upstream enhancer, in addition to other GnRH regulatory elements (Givens et al., 2004). Slower mobility complexes that bind the upstream enhancer contain Oct-1 and also seem to associate with NF1, suggesting a functional complex containing these two proteins that may interact at the level of GnRH regulatory sequence. Additional studies in our laboratory confirmed that NF1 and Oct-1 could physically interact *in vitro* through GST pull-down assays (Givens et al., 2004).

While transcripts of all four NF1 family members (NF1A, NF1B, NF1C, and NF1X) are expressed in our GnRH model cell lines, data suggest that NF1C may be the functional protein in GnRH gene regulation. The Nuclear Factor 1 family also contains several splice variants (Kruse and Sippel, 1994), and while several spice

variants of each NF1 were found in our GnRH neuron model cell line, further analysis will be necessary to determine what specific roles they might be playing.

Furthermore, our findings suggest that NF1 functionally interacts with Oct-1. The characteristic Oct-1 activation of GnRH promoter activity was diminished by over-expression of a dominant-negative NF1. Functional interaction between NF1 and Oct-1 has been reported to regulate genes such as MMTV (O'Connor and Bernard, 1995) and HPV-16 (Hebbar and Archer, 2003). Interestingly, in these systems, as in ours, the NF1 binding sites are in close proximity to the Oct-1 binding sites. In fact, we previously demonstrated that by mutating the NF1 binding sequence, Oct-1 binding is reduced and mutating the Oct-1/NF1 sequences in the context of reporter assays, drastically decreased GnRH promoter activity (Givens et al., 2004). These findings indicate an important role for NF1 in GnRH gene regulation and confirm the significance of the upstream enhancer as a GnRH regulatory element.

Completion of the rat genome sequence allowed a more extensive analysis of the upstream GnRH sequence. While previous research only had use of approximately -3 kb relative to the GnRH start site, the complete rat genome has since become available. The -5 kb rat, mouse, and human sequences were compared for homology. Four novel regions emerged from this analysis. All four have high sequence homology, ranging from 80% (region 1) to 96% (region 2), suggesting evolutionary significance. Importance of the additional upstream 2 kb was demonstrated by the increase in activity of the full-length -5 kb rat GnRH luciferase over the reporter containing the upstream enhancer, enhancer, and promoter. 5’ truncation analysis

highlighted the importance of regions 1 and 3 for GnRH reporter activity. Adding region 1 to the 5' end of the upstream enhancer resulted in a significant enhancement of the promoter. However, this was not observed when the upstream enhancer alone was used and therefore reinforces the hypothesis that region 1 is a continuation of the characterized upstream enhancer which was not discovered initially due to the lack of rat genomic sequence available to the public at the time. Similarly, region 3 also appears to be an important cis-regulatory element for GnRH promoter activity. When eliminated, promoter activity is reduced and inserting region 3 increases promoter activity over the promoter or enhancer/promoter alone. Current studies in our laboratory also demonstrate that regions 1/UE and 3 display classic characteristics of true enhancers, such as functioning in both forward and reverse orientation and ability to enhance activity of a heterologous promoter (A.K. Iyer & P.L. Mellon, personal communication).

In silico analysis reveals many potential transcription factors consensus binding sites within regions 3 and 1. Of these, Oct-1 is known to be an essential regulator of the GnRH gene (Chandran et al., 1999; Clark and Mellon, 1995; Wolfe et al., 2002). Oct-1 (and its binding partner NF1) bind multiple sites within previously characterized regulatory elements. Oct-1 also binds several other proteins that are known to regulate GnRH such as C/EBP (Belsham and Mellon, 2000), TALE family members Pbx and Prep (Rave-Harel et al., 2004), and Groucho-related gene family members (Rave-Harel et al., 2005). *In vitro* experimental evidence reveal two additional binding sites for Oct-1 within regions 1 and 3. We also demonstrate that

Pbx and Prep family members can bind to region 4. While region 4 did not significantly contribute to basal GnRH gene transcription, it may play a role in hormonal regulation of GnRH or the circadian regulation of GnRH.

Region 2 is a short (31 bp) but highly conserved sequence. While deletion of region 2 did not reduce GnRH promoter activity as did regions 1 and 3, it did result in a significant activation ($p<0.05$), suggesting the loss of a negative regulatory factor (Figure 2-8). *In silico* analysis using MatInspector and Transfac revealed several potential transcription factor binding sites that would be very interesting to explore in future experiments.

It is of interest to note that transient transfection of the -5 kb or addition of the novel regulatory regions 1/UE and 3 to previously characterized regulatory elements, specifically increased promoter activity in GT1-7 cells, but not in Gn11 cells, the GnRH model representing a less mature GnRH neuron. As Gn11 cells express very low levels of GnRH, we would expect genuine GnRH regulatory elements to be less or inactive in these cells. These results indicate that these additional conserved regulatory elements contribute to GnRH neuron-specific expression of GnRH.

In summary, we have identified novel regulators of the GnRH gene. NF1 binds GnRH regulatory elements in close proximity to Oct-1 and appears to function by affecting Oct-1 regulation of GnRH. Four novel evolutionarily-conserved regions were identified upstream of the characterized regulatory elements. Two of these, regions 1 and 3, are important for GnRH neuron-specific GnRH transcriptional activity, and are capable of binding transcription factors that are known to regulate

GnRH gene expression. These finding represent new information that may contribute to the knowledge of how the precise expression of GnRH gene is controlled.

MATERIALS AND METHODS

Cell Culture and Transfections. GT1-7, Gn11, and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium with 4.5% glucose, 10% fetal bovine serum and 1x penicillin-streptomycin in 5% CO_2 at 37°C. GT1-7, Gn11, and NIH3T3 cells were split into 24-well plates for transfection at 90,000, 50,000, and 25,000 cells per well, respectively. Transient transfections were performed using FuGENE 6 Transfection Reagent (Roche) according to manufacturer's protocols. 400 ng/well of rat GnRH-luciferase vectors or empty pGL3 vector were co-transfected with 100 ng/well of thymidine kinase-β-galactosidase as an internal control. For co-transfection experiments, 100 ng/well of Oct-1 or NF1 expression vector were used.

Cells for reporter assays were harvested 48 hours after transfection in lysis buffer (100 mM potassium phosphate and 0.2% Triton-X-100, pH7.8). Luciferase assays were performed as previously described (Givens et al., 2005) and β-galactosidase assays were performed as directed by the manufacturer (Tropix, Bedford, MA). Luciferase values were normalized to β-galactosidase values as internal control, to control for transfection efficiency. Additionally, transient transfections performed on multiple cell lines were co-transfected with a RSV-luciferase reporter in order to control for the differences in transfection efficiency and metabolism between the cell lines. Values were always compared to empty vector control. Experiments were performed in quadruplicate and repeated a minimum of

three times. Data represent the mean, ± SEM of at least three independent experiments, each performed in quadruplicate.

Nuclear Extracts and EMSA. Nuclear extracts for EMSA were prepared according to Lee *et al.* (Lee et al., 1988). Annealed oligonucleotides (1 pmol) (QIAGEN, Alameda, CA) were phosphorylated with [γ^{32}P]ATP (6000 Ci/mmol; ICN Biomedicals, Inc., Aurora, IL) and T_4 polynucleotide kinase (New England Biolabs, Inc, Beverly, MA). The probes were purified using MicroSpin G-25 columns (Amersham Biosciences Corp., Piscataway, NJ). Each binding reaction contained 1 fmol probe and 2 or 10 µg nuclear extracts in 10 mM HEPES (pH 7.8), 50 mM KCl, 1 mM EDTA, 5 mM spermidine, 5 µg BSA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride, 0.5 µg poly dI-dC, 6% glycerol, and 2% Ficoll. The binding reaction was incubated 5–30 min at room temperature after addition of probes. The DNA-protein complexes were electrophoresed for 2 hours at 250 V and separated on 5% acrylamide-*N*,*N*-methylene bisacrylamide (30:1) gels, then dried under a vacuum and exposed to film for 1–3 days. For competition reactions, 100 fmol (100x labeled probe) of unlabeled annealed double-stranded oligonucleotides were added to the reaction. Supershift assays were performed with 0.2 µg of anti-OCT-1 antibody (Santa Cruz Biotechnology., Santa Cruz, CA), 1 µl of anti-NF1 antibody (kindly provided by Dr. Naoko Tanese), 1 µl anti-Pbx or anti-Prep antibody (Santa Cruz Biotechnology, Inc). The NF1 consensus oligonucleotide sequence is 5'-AGTTTGGCAGGGAGCCAAGTT-3'. The Pbx consenus oligonucleotide is

5'-AGCGCGGGGCGCATCAATCAATTTCG-3'. Additional EMSA oligonucleotide probe sequences are described in Figure 2-11.

RT-PCR. Total RNA extraction was carried out as described by the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The reverse transcriptase reaction was performed according to the methods of Pernasetti *et al*. (Pernasetti et al., 2001). cDNA was obtained by reverse transcribing 5 μg RNA from GN11, NLT, GT1–7, NIH3T3, and LβT2 cells using Oligo$(dT)_{12–18}$ primer and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR analysis was performed with 35 cycles of 45 sec at 95°C, 1 min at an annealing temperature of 60°C, and 1 min at 72°C, followed by an elongation step for 10 min at 72 C. Specific primers for *NFIA*, *NFIB*, *NFIC*, *NFIX*, and β-*actin* were used in the PCR reaction. Primers for the *NFI* genes were created by the Gronostajki Lab, http://elegans.swmed.edu/Worm_labs/Gronostajski/ and amplify regions of the *NF1* sequences that are not alternatively spliced. PCR products were viewed by electrophoresis on an ethidium-stained, 1.5% agarose gel.

Western Blot Analysis. Western blotting was performed using nuclear extract prepared from GT1-7 cells over-expressing NF1 proteins. To obtain nuclear extract with over-expressed proteins, cells were plated on 10cm culture dishes and transiently transfected with 5 μg of plasmid expression vector for HA-tagged NF1A, NF1B, NF1C, and NF1X, using the method described previously. Nuclear protein was

harvested 48 hours after transfection (Lee et al., 1988). 12 µg protein was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane. After blocking in 5% milk, antibodies recognizing NF1 (Santa Cruz Biotechnology, Inc.) or haemagglutinin (HA) tag (Roche) were incubated at 1:1000 and 1:1500, respectively, at room temperature for 1 hour. Secondary antibodies conjugated to horseradish peroxidase were used to detect signal and visualized using the ECL kit (Amersham) according to manufacturers' protocol.

Sequence analysis. Approximately 5 kb of the sequence 5' to the GnRH start site for the rat, mouse, and human sequence were obtained through the NCBI database and used for analysis. Sequences were aligned by a pairwise BLAST using the following parameters: blastn matrix, 1, _2; extension, 2; gap x_dropoff, 50; expect, 10; wordsize, 11. The low resolution image was reproduced in Microsoft Office PowerPoint 2003. Alternatively, VISTA analysis (Frazer et al., 2004) was performed using the rat chromosome 15: 47,314,415 to 47,318,982 as the base genome, aligning with human and mouse sequence. *In silico* transcription factor binding site analysis was performed using Genoatrix MatInspector (Quandt et al., 1995), TRANSFAC, and TESS publically available software.

Molecular Cloning and Mutagenesis. The -5 kb (-4984 to +22 relative to start site) GnRH sequence was isolated and amplified from rat genomic DNA using the F1 and R2 -5 kb PCR primers described in Table 2-1. The fragment was cloned into the

pCR2.1 vector using the TOPO cloning kit (Invitrogen). KpnI and XhoI restriction sites were used to insert the fragment into the pGL3 luciferase expression vector (Promega). Ligations were performed using T4 DNA Ligase (NEB).

Truncations were created by either molecular cloning using the -5 kb rat GnRH in pGL3 as a template or by targeted mutagenesis generating deletions in the -5 kb rat GnRH-luciferase vector. The -3620, -3175, -2438, -2168, -1380, and -957 rat GnRH-luciferase vector truncations were made using a common 3' and specific 5' primer and individually PCR amplifying, TOPO cloning, and ligating into KpnI and XhoI restriction sites in the pGL3 backbone as described above. The Quick Change Site Directed Mutagenesis kit (Stratagene) was used to create the -4491 and -4199 rat GnRH-luciferase reporters by targeted deletions. The -4491 plasmid was made using the -5 kb as template and then the -4491 was subsequently used as template for the -4199. Sequences for PCR primers used for truncations are described in Table 2-1.

Reporter vectors containing regions 1/UE and/or region 3 with rat GnRH enhancer and promoter elements were created by amplifying the respective regions from the -5 kb full length rat GnRH plasmid using #3 and #1/UE forward and reverse PCR primers described in Table 2-1, then transferred into KpnI and MluI sites in the GnRH-UE/E/P, GnRH-E/P, and GnRH-P reporter vectors previously described (Givens et al., 2004; Kelley et al., 2002).

Table 2-1. Sequences of Primers

Primer	Sequence (5'-3')
F1 -5 kb	GGGGTACCCCTGGAACCTGAGTTCAGATCATTGTGAGCTC
R3 -5 kb	CCGCTCGAGCGGGCTGGTGACCATAGTGCAGCGTAGACCATA
3'trunc	GGGCTAGCCCGCTGGTGACCATAGTGCAGCGTAGACCATA
5' -957	GGGGTACCCCGAACACTGAGCAATGACGGTAGGAGTGTTG
5' -1380	GGGGTACCCCCCACTTGTTAGTCCTAGCCAGAAACCACAG
5' -2168	GGGGTACCCCGTCAGGCTCTTATGTAGTCAAGTTCCTCGG
5' -2438	GGGGTACCCCCTAGGGAAGACACCCTGTGACTTGTATTGC
5' -3175	GGGGTACCCCCTTTACCTTCCTAGTGGATGTCCCAGTGTC
5' -3620	GGGGTACCCCCTGTCCCGTCACTAGTTAGTTCTCATGCTC
-4491 fwd	TTTCTCTATCGATAGGTACCTCTTGCAGAACTGTGATTTG
-4491 rev	CAAATCACAGTTCTGCAAGAGGTACCTATCGATAGAGAAA
-4199 fwd	TTCTCTATCGATAGGTACCGTAACAGAAGAACACTCTG
-4199 rev	CAGAGTGTTCTTCTGTTACGGTACCTATCGATAGAGAA
#3 fwd	GGGGTACCCCTAACAGAAGAACACTCTGTTCATAGATGTA
#3 rev	CGACGCGTCGAGGCATTACTAAAGCTATTTATAAACCCGA
#1/UE fwd	GGGGTACCCCCCCAAGAGCAGGGAAGAAGCTGTGTCCAAA
#1/UE rev	CGACGCGTCGCCGAAACAGCCGGTCAGCGGTCCACACCAT

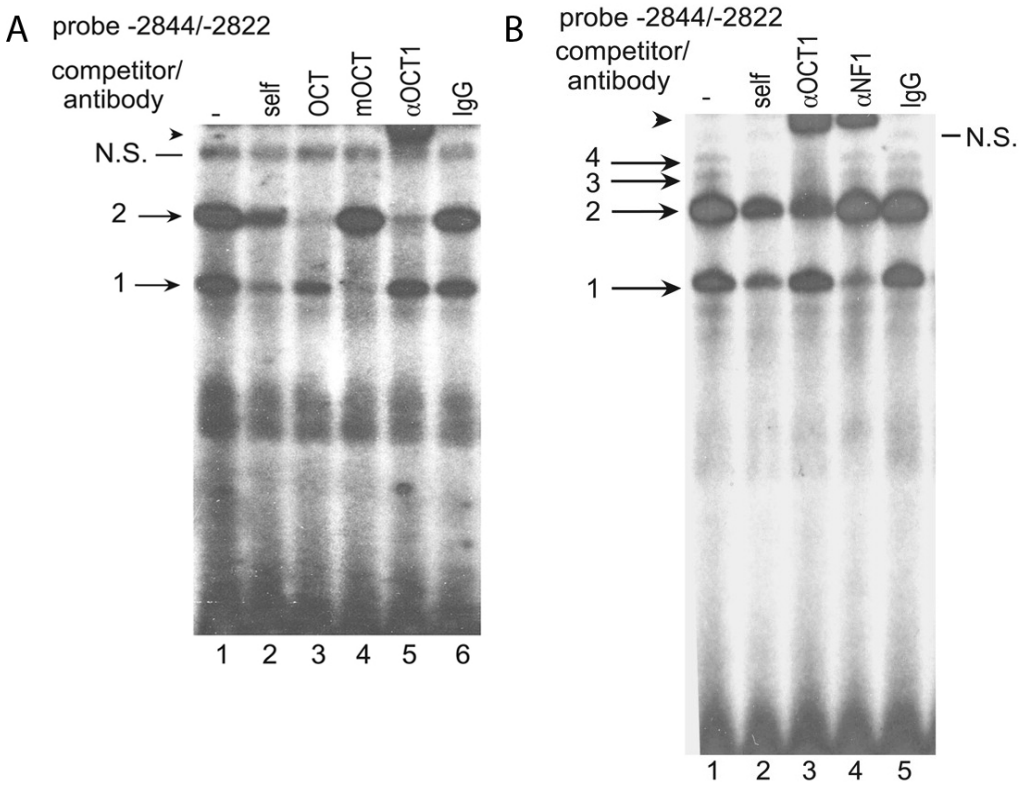

Figure 2-1. Nuclear Factor 1 binds the upstream enhancer. A) EMSA analysis was performed using the -2844/-2822 radiolabeled probe and 2 µg GT1-7 nuclear extract. Lane 1 shows protein complex binding to the DNA, lane 2 is self-competition, lane 3 represents competition with an Oct-1 consensus sequence, lane 4 is competition with the mutant Oct-1 oligonucleotide, lane 5 shows the Oct-1 complex (arrow 2) super-shift with an anti-Oct-1 antibody, and an IgG control is shown in lane 6. Arrow 2 marks the faster-mobility complex described. N.S. indicates non-specific binding. The arrowhead marks the super-shifted proteins. B) EMSA using 10 µg of GT1-7 nuclear extract. Four major complexes form, represented by arrows (1-4). Lane 1 shows the complex formation, lane 2 shows self-competition, antibodies recognizing Oct-1 and NF1 are shown in lanes 3 and 4, respectively, and an IgG control is shown in lane 5. The arrowhead marks the super-shifted complexes.

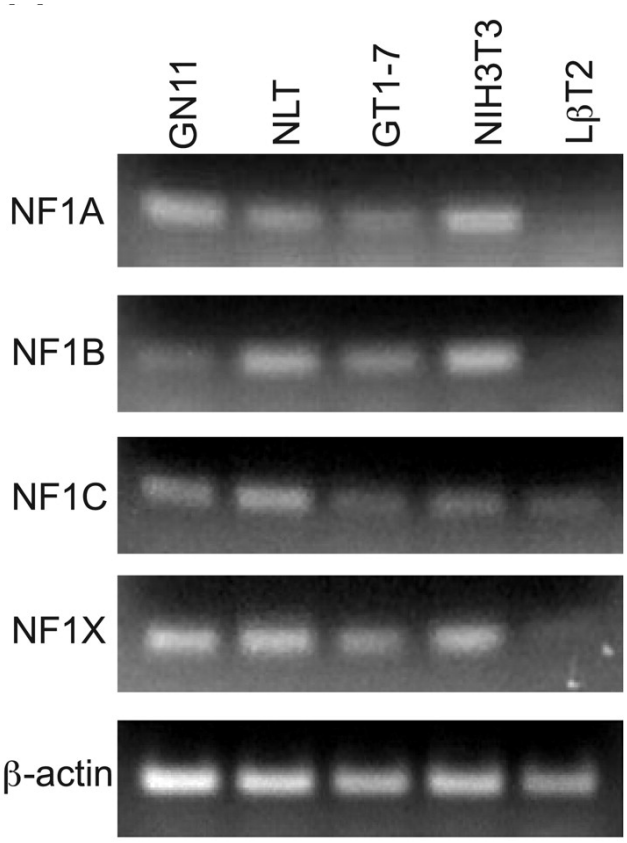

Figure 2-2. Expression of the NF1 family members. RT-PCR was performed using total RNA from Gn11, NLT, GT1-7, NIH3T3, and LβT-2 cell lines. PCR products are shown run on a 1% agarose gel stained with ethidium bromide. Primers for each NF1 family member correspond to the unspliced, N-terminal region of the cDNA. β-actin was used to control for equal loading.

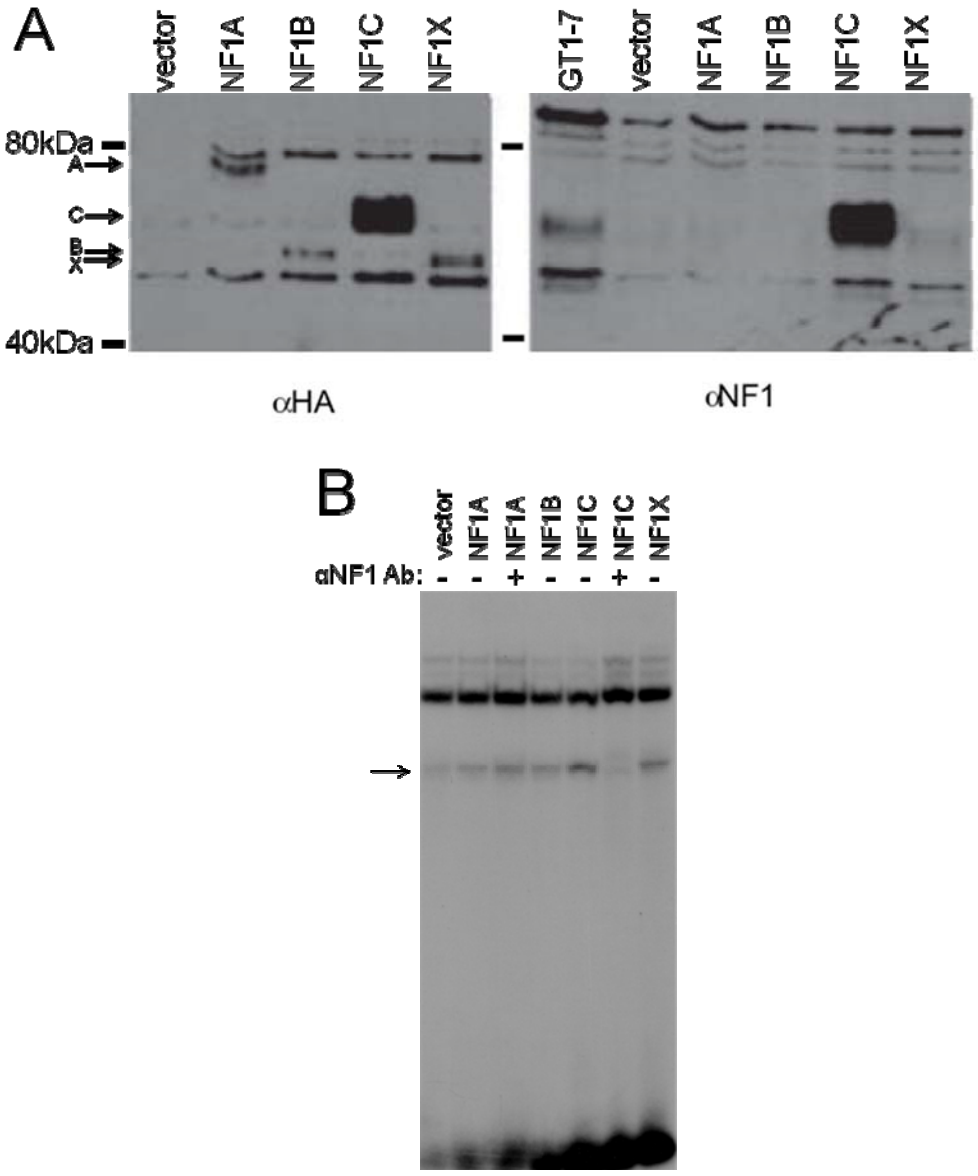

Figure 2-3. NF1 protein expression in GT1-7 cells. A) Western blot analysis of NF1 proteins endogenously or over-expressed in GT1-7 cells. Arrows indicate overexpressed proteins recognized. B) EMSA analysis using -2844/-2822 probe and 2 µg GT1-7 nuclear extract. Over-expressed proteins and presence of NF1 antibody are indicated along top. Arrow marks NF1 complex whose binding is disrupted by the αNF1 antibody.

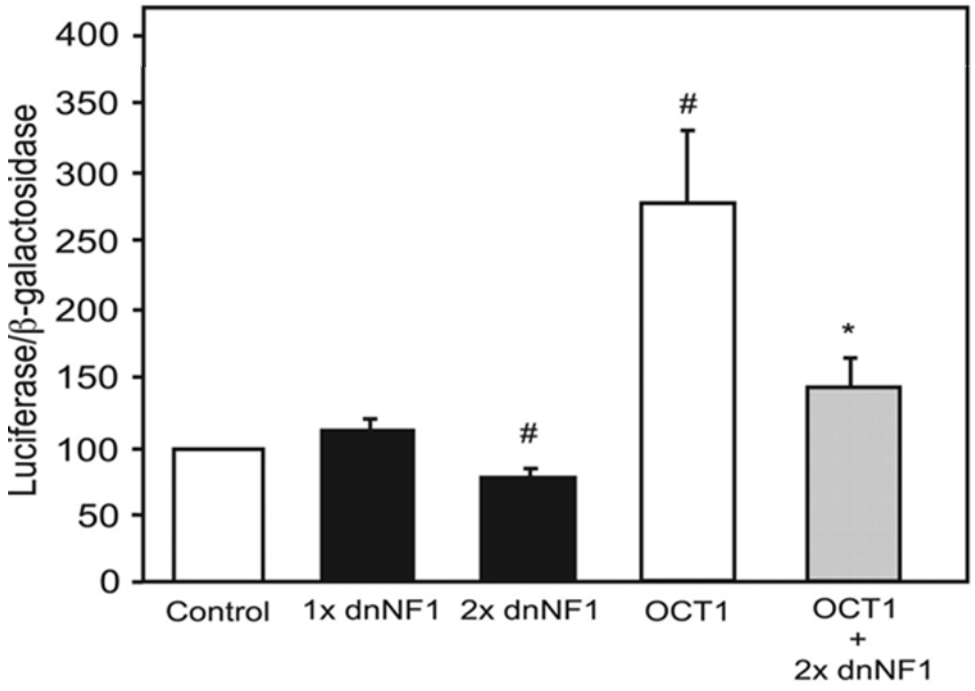

Figure 2-4. NF1 and Oct-1 functionally interact. Transient transfections were performed in GT1-7 cells using a multimer of an Oct-1 binding sequence in the GnRH enhancer (-1805 to -1766) fused to the RSV promoter driving luciferase expression. 100 ng of dominant negative NF1/engrailed fusion (dnNF1) is 1X. Luciferase values were normalized to β-galactosidase to control for transfection efficiency and vector-only control was set to 100% activity. # indicates statistical difference from the expression vector-only control. * indicates statistical difference between Oct1 and Oct1 plus the dominant negative.

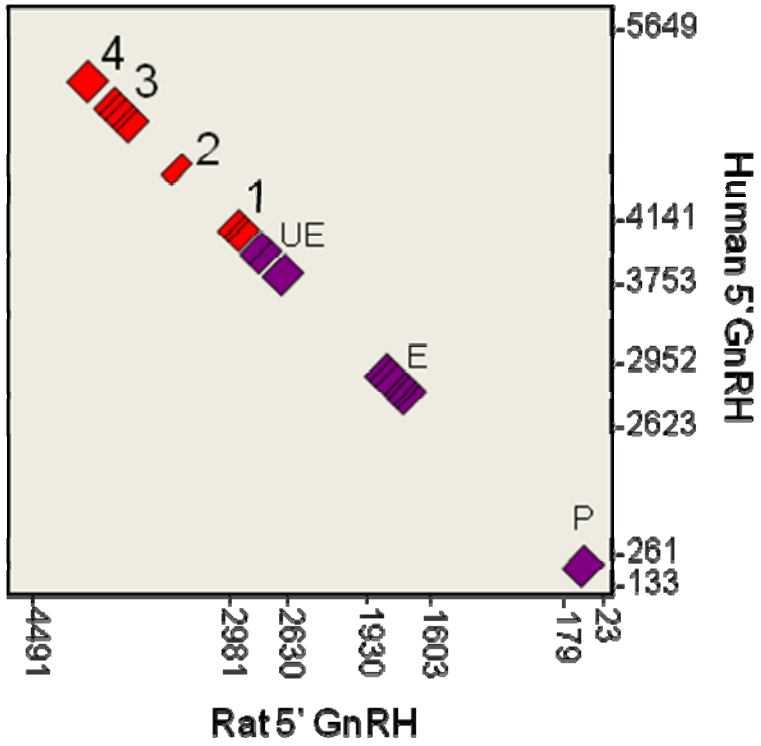

Figure 2-5. Identification of far-upstream conserved regions. Pairwise BLAST was used to compare the 5 kb upstream of the rat GnRH start site (X-axis) to the 5 kb upstream of the human GnRH start site (Y-axis). Four novel conserved regions, colored red and labeled 1-4 are revealed. UE, upstream enhancer; E, enhancer; P, promoter.

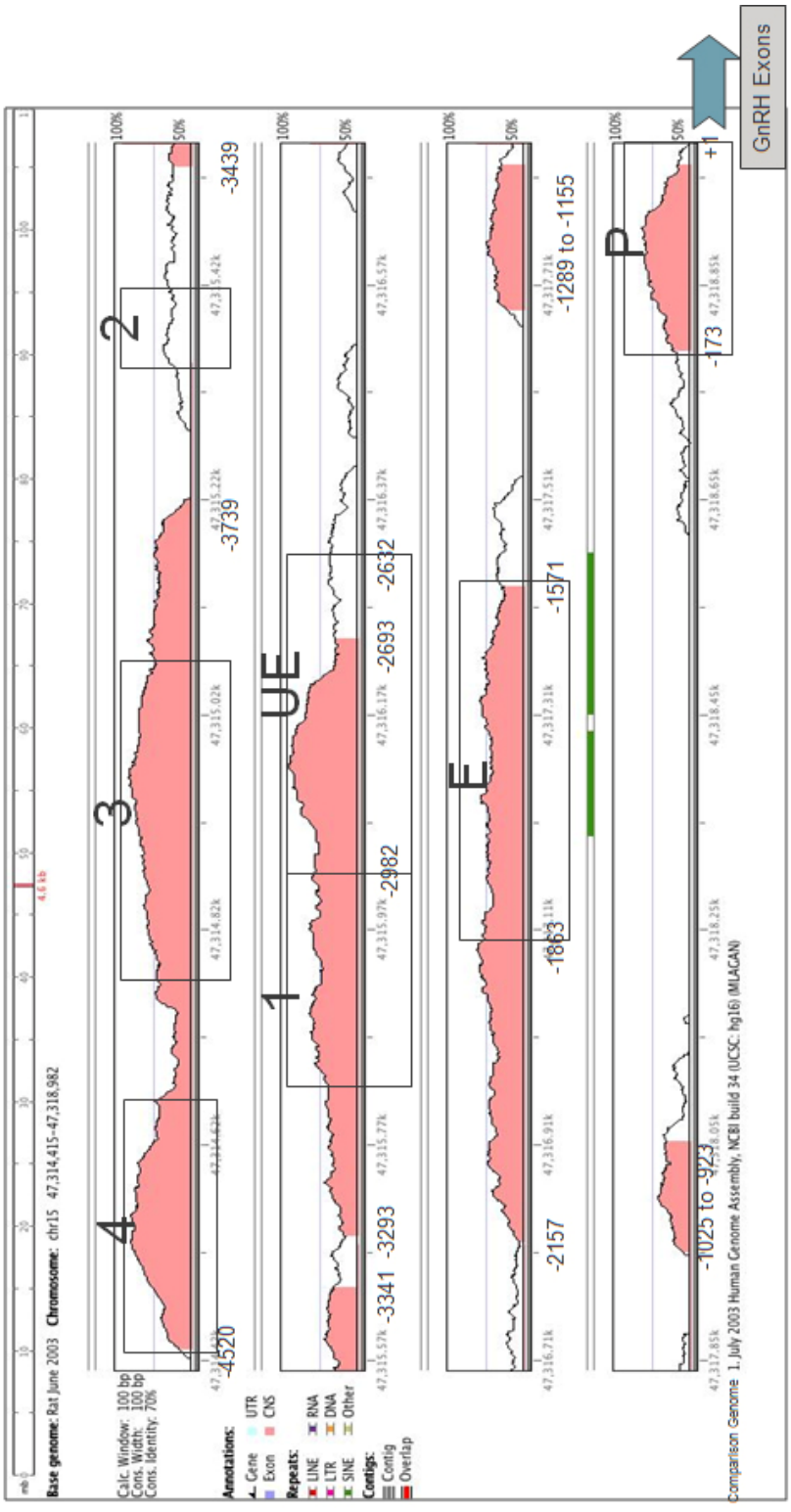

Figure 2-6. VISTA plot analysis using the rat genome as base sequence and comparing to human sequence 5 kb upstream of the GnRH start site are shown. Pink coloring indicates homologous regions and the height of the peaks within these regions are % homology. Regions identified by BLAST in Figure 2-5 are indicated with black boxes and the appropriate labels. UE, upstream enhancer; E, enhancer; P, promoter

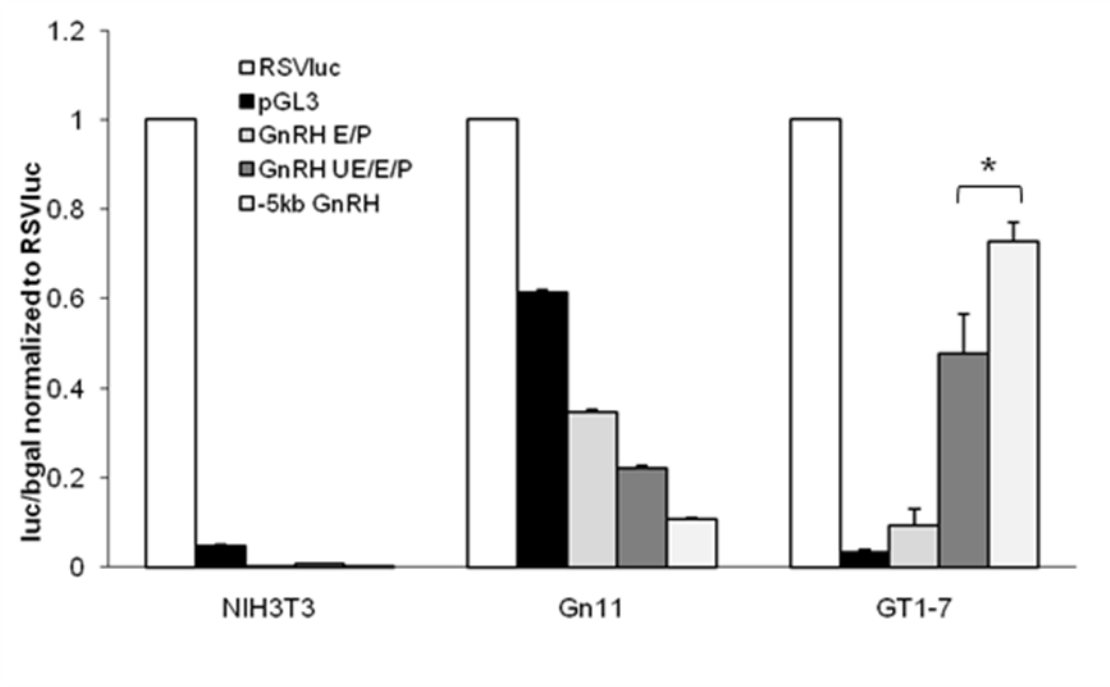

Figure 2-7. The -5 kb GnRH regulatory region confers specificity over the previously characterized GnRH regulatory regions alone. Transient transfections were performed in GT1-7, Gn11, and NIH3T3 using luciferase reporter plasmids containing the -5 kb GnRH or the upstream enhancer (UE), enhancer (E), promoter (P). RSVluc plasmid was transfected in parallel to control for differences in transfection efficiency between the cell lines. pGL3 is the empty luciferase vector. Error bars indicate the SEM. * indicates significant difference between the two bars.

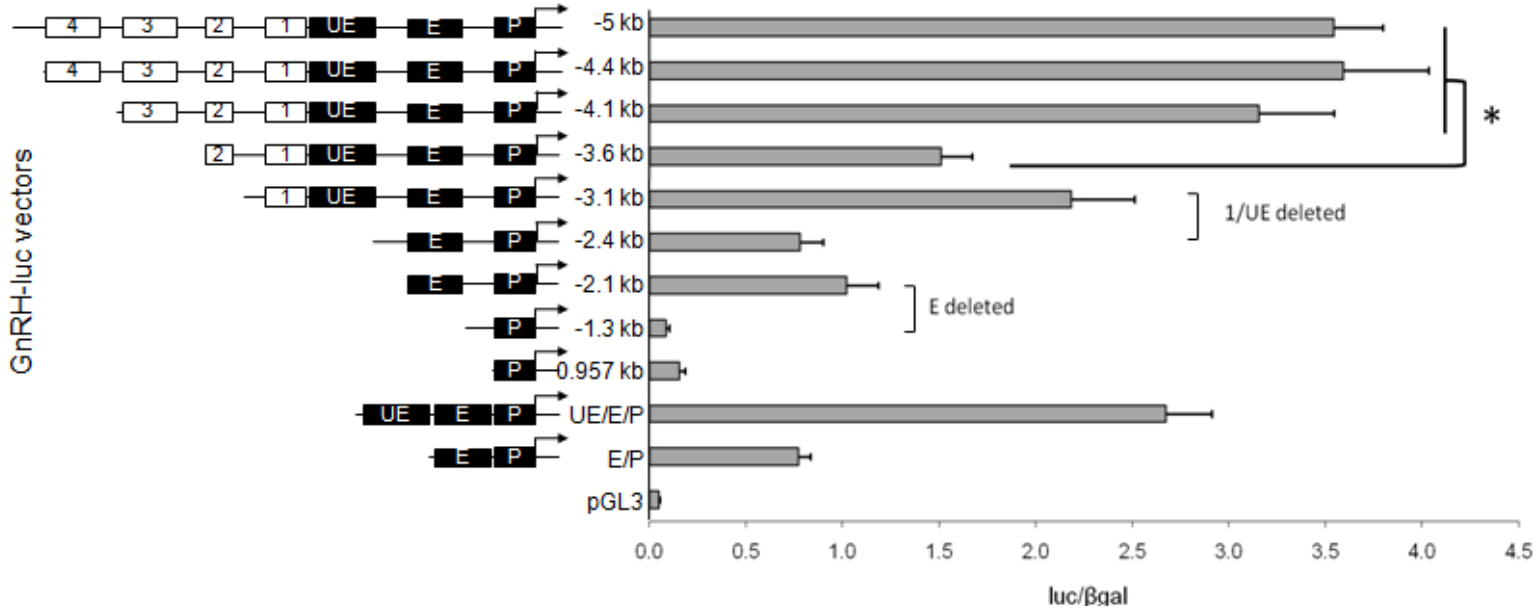

Figure 2-8. Truncation analysis identifies critical regions for basal GnRH gene expression. Transient transfections were performed in GT1-7 cells using 5' truncations of the -5 kb GnRH-luciferase reporter. Diagrams on the left depict the truncations and the conserved regions that they contain. Also shown are the GnRH-UE/E/P and GnRH-E/P for comparison and the pGL3 empty luciferase vector for control. The bar graph on the right shows the relative reporter activity of the truncations. Values are luciferase normalized to β-galactosidase activity as an internal control. Error bars are SEM. * indicates significance by one-way ANOVA an Tukey-Kramer HSD. UE, upstream enhancer; E, enhancer; P, promoter

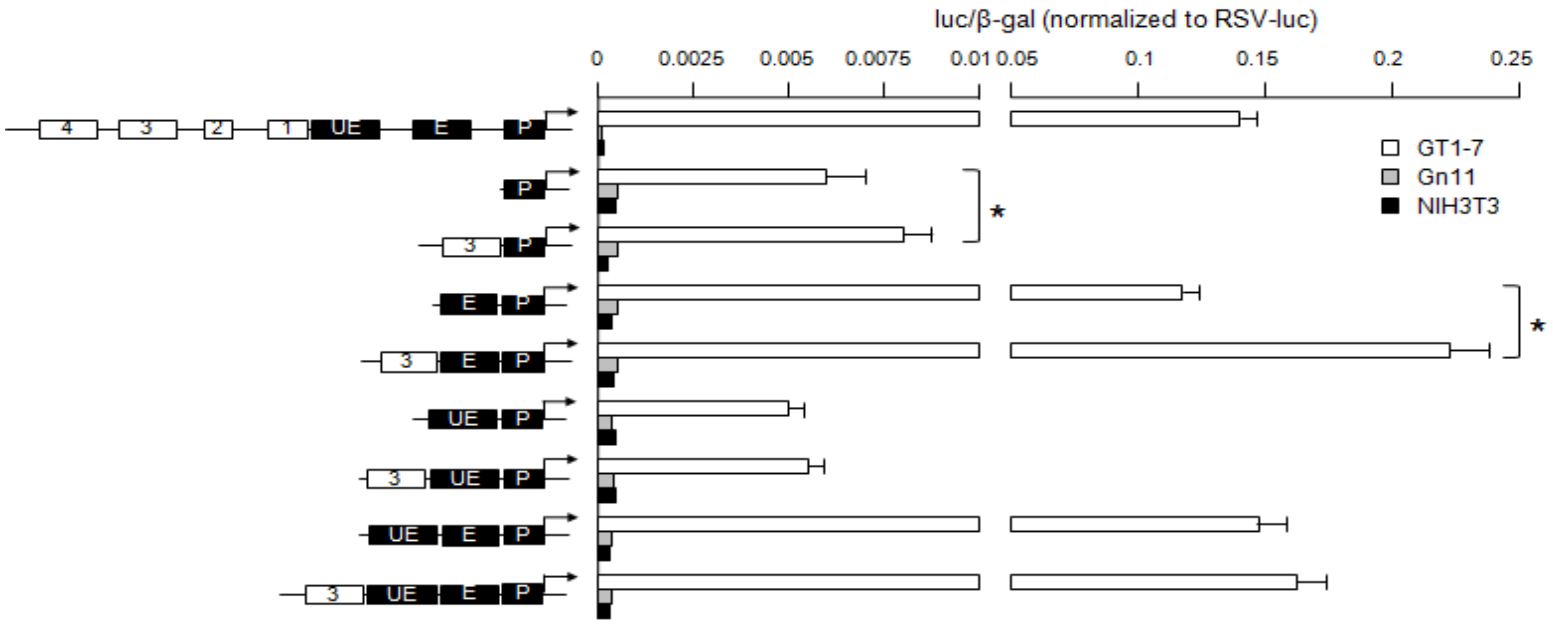

Figure 2-9. Region 3 enhances GnRH promoter activity. Transient transfections were performed in GT1-7, Gn11, and NIH3T3 cells using luciferase reporter plasmids containing the novel conserverd region 3 and the previously characterized GnRH regulatory elements , P – promoter, E – enhancer, UE – upstream enhancer. The -5 kb reporter c ontaining all regulatory elements is shown for control. β-galactosidase was used as an internal control and luc/β-gal values were normalized to RSV-luc to control for transfection efficiency between the cell lines. * indicates significant difference by one-way ANOVA and Tukey-Kramer HSD.

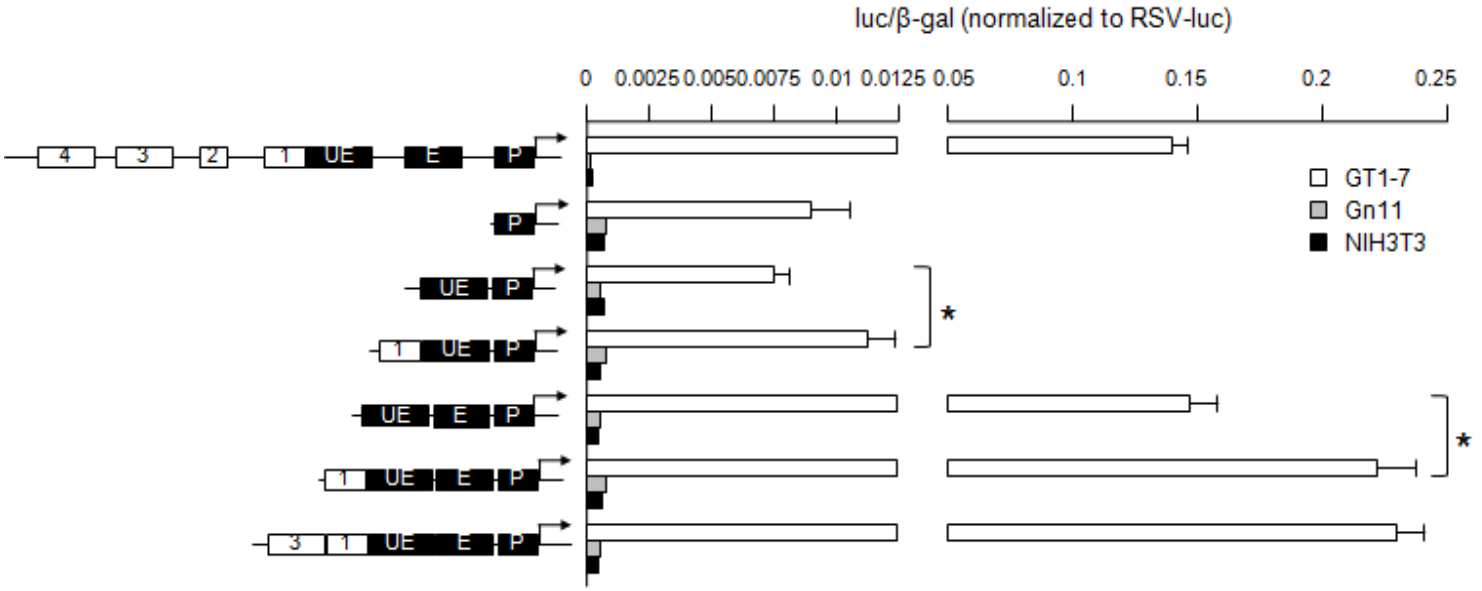

Figure 2-10. Region 1 contributes to the activity of the characterized upstream enhancer. Transient transfections were performed in GT1-7, Gn11, and NIH3T3 cells using luciferase reporter plasmids containing the novel conserverd region 1 and the previously characterized GnRH regulatory elements , P – promoter, E – enhancer, UE – upstream enhancer. The full length -5 kb reporter c ontaining all regulatory elements is shown for control. β-galactosidase was used as an internal control and luc/β-gal values were normalized to RSV-luc to control for transfection efficiency between the cell lines. * indicates significant difference by one-way ANOVA and Tukey-Kramer HSD.

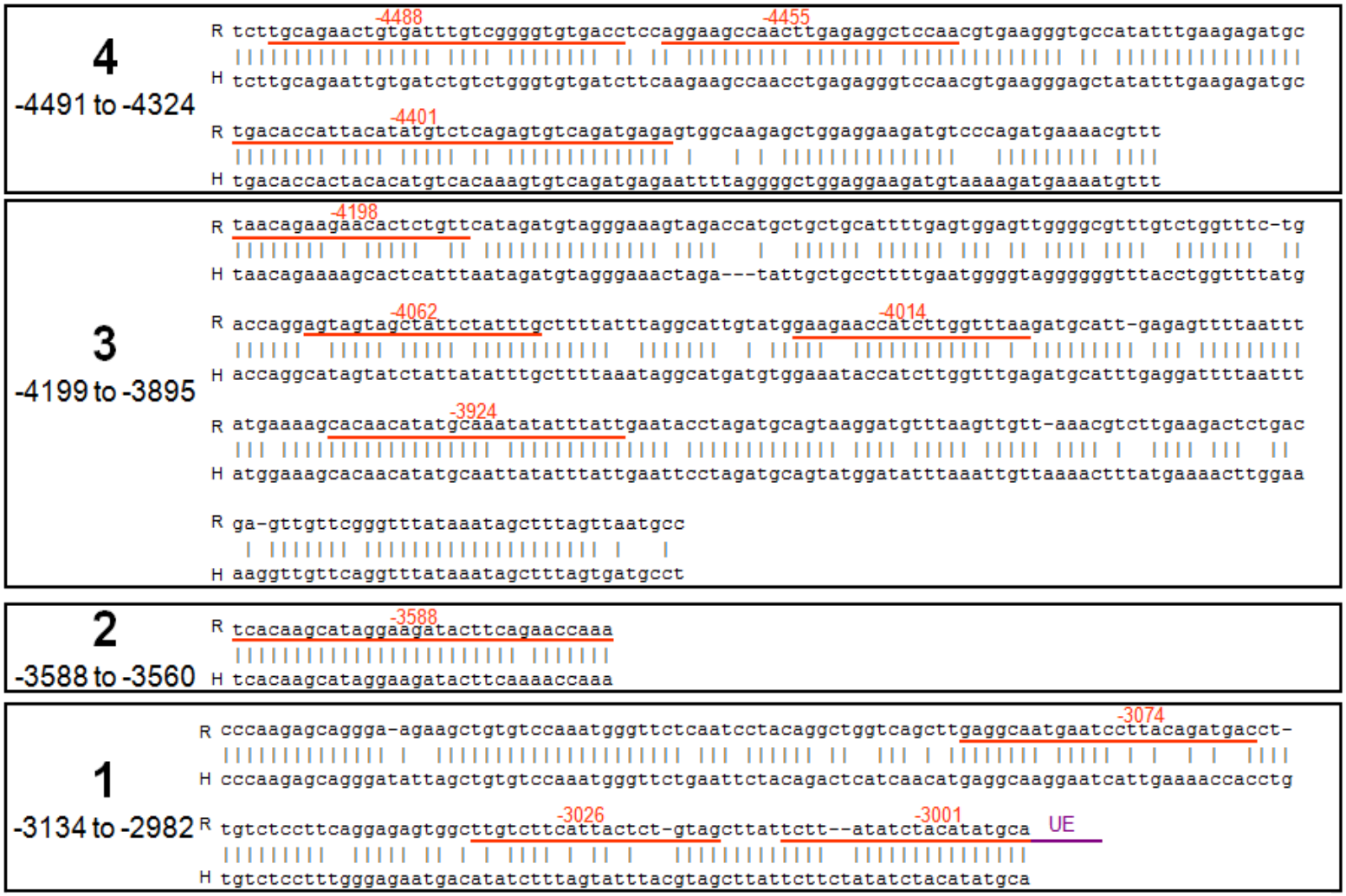

Figure 2-11. Sequence alignment of the evolutionarily-conserved regions. R is rat genomic sequence and H is human genomic sequence. Red underlining and numbering refer to EMSA probes used in Figure 2-12.

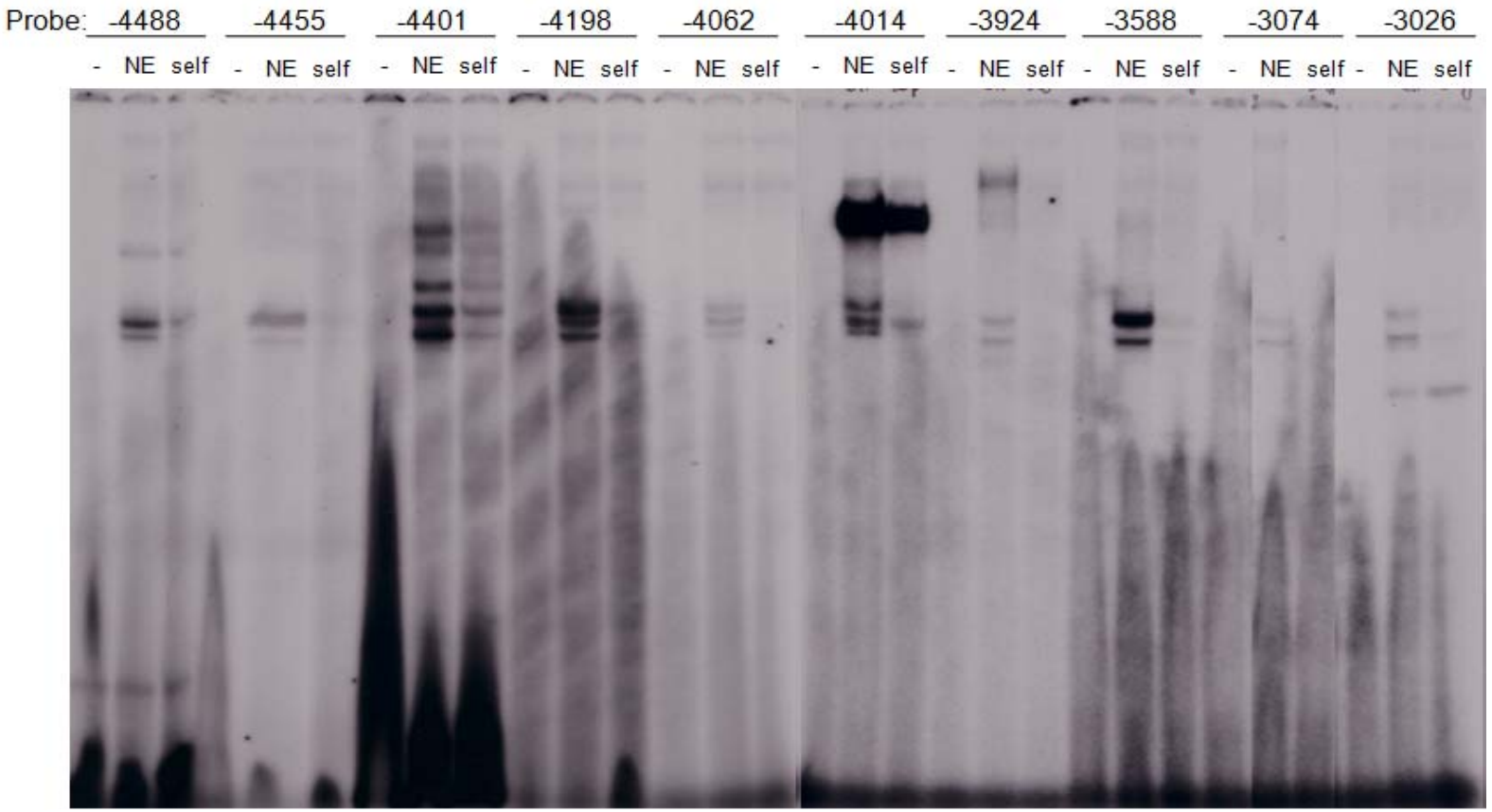

Figure 2-12. Proteins bind to all four conserved regions. EMSA analysis was performed using GT1-7 nuclear extract and radiolabeled oligonucleotides probes corresponding to various regions upstream of the rat GnRH gene transcription start site. Probe numbers correspond to sequence depicted in Figure 2-11. For each probe, probe alone control is shown in the first lane, protein complex formation is shown in the second lane, and 100x unleabled oligonucleotde self-competition is shown in the third lane.

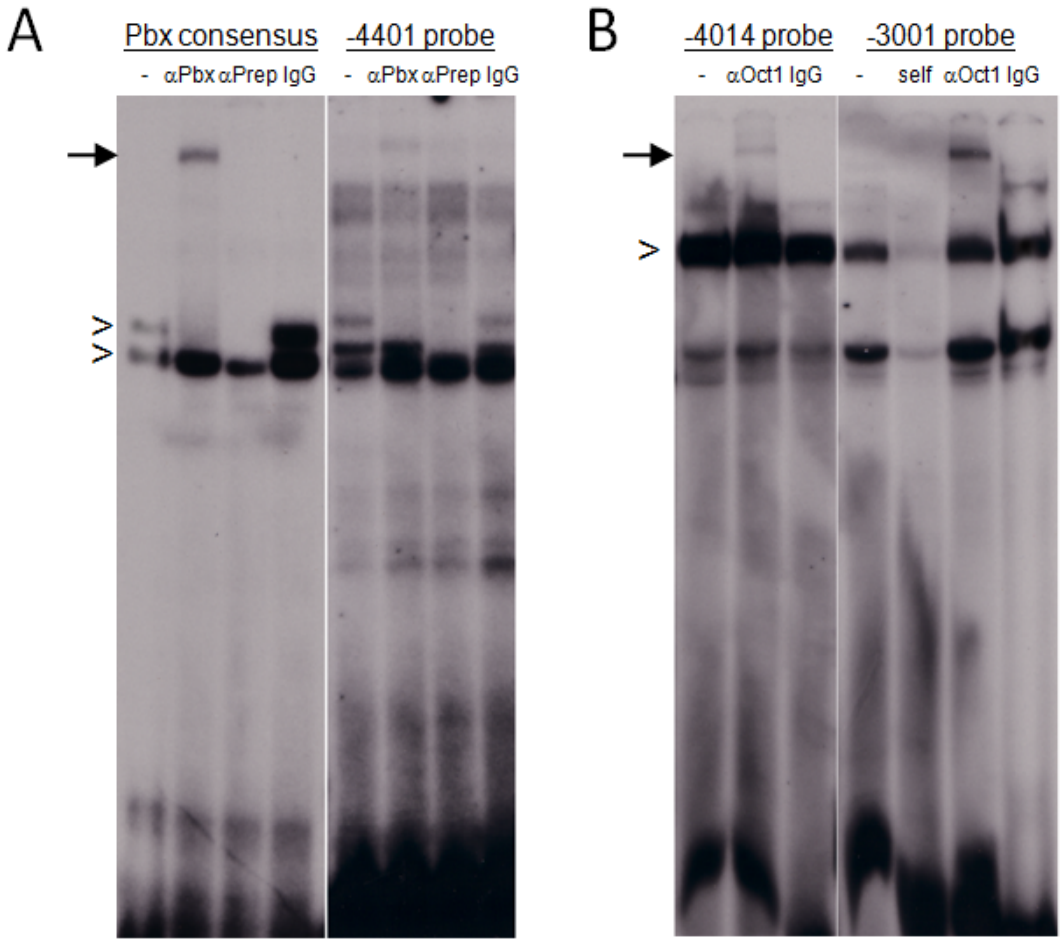

Figure 2-13. Known GnRH regulators, Pbx/Prep and Oct-1, bind to the novel conserved regions. A) EMSA analysis and antibody supershifts were performed using GT1-7 nuclear extracts and a Pbx consensus probe (left) and a oligonucleotides probe corresponding to -4401 of the rat GnRH gene (right). In both cases, lane 1 shows protein complex formation, lane 2 includes anti-Pbx antibody, lane 3 incldues an anti-Prep antibody, and lane 4 is an IgG control. Open arrowheads mark the Pbx and Prep protein complexes. Arrow represents the anti-body supershift. Note that the anti-Prep antibody does not produce a super-shift but rather disrupts binding of the protein to the DNA. B) EMSA analysis was performed using oligonucleotide probes corresponding to -4014 and -3001 of the rat GnRH gene and GT1-7 nuclear extract. For the -4014 probe, lane 1 depicts the protein complexes formed on the probe, lane 2 includes an anti-Oct-1 antibody, and lane 3 is an IgG control. Using the -3001 probe, lane 1 is protein complex formation, lane 2 shows 100x unlabeled oligonucleotides self competition, lane 3 includes anti-Oct-1 antibody, and lane 4 is an IgG control. Open arrowhead represents Oct-1 protein complex and arrow represents the antibody super-shift.

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A portion of the material in Chapter II is from the publication "Phylogenetic Footprinting Reveals Evolutionarily Conserved Regions of the Gonadotropin-Reseasing Hormone Gene that Enhance Cell-Specific Expression" Marjory L. Givens, Reiko Kurotani, Naama Rave-Harel, Nichol L. G. Miller, and Pamela L. Mellon. Molecular Endocrinology, 2004 18(12): 2950-2966. Copyright permission has been granted by the owner, The Endocrine Society. The dissertation author contributed substantial research and critical reading of this paper.

A portion of Chapter II is, in part, a manuscript in preparation titled "Evolutionarily Conserved Regions of the Gonadotropin-releasing Hormone Gene Enhance Neuronal Specification to Differentiated Neurons" by Nichol L. G. Miller, Anita K. Iyer, and Pamela L. Mellon. The dissertation author is an equally contributing primary researcher of this manuscript. Anita K. Iyer contributed molecular cloning and transfections. All other work contained within, including the writing of this portion was done by Nichol L. G. Miller.

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CHAPTER III

Necdin, a Prader-Willi Candidate Gene, Regulates Gonadotropin-Releasing Hormone Gene Expression

Prader-Willi syndrome (PWS) is a complex genetic disorder characterized by hyperphagia, obesity, and hypogonadotrophic hypogonadism, all highly suggestive of hypothalamic dysfunction. The *NDN* gene, encoding the MAGE family protein, necdin, maps to the PWS chromosome region and is most highly expressed in mature hypothalamic neurons. Necdin interacts with homeodomain proteins, Msx and Dlx, through another MAGE family member, Dlxin. Our laboratory has shown that Msx and Dlx transcription factors contribute to GnRH gene transcription and GnRH neuronal development. Herein, we show that high levels of necdin are present in a mature GnRH neuronal cell line, and it is not detected in an immature migratory GnRH neuronal cell line. Furthermore, overexpression of necdin activates GnRH transcription, acting through enhancer and promoter elements bound by the homeodomain repressor Msx. In fact, overexpression of Necdin relieves Msx repression of GnRH transcription and necdin coimmunoprecipitates with Msx from GnRH neuronal cells indicating that necdin may activate by preventing Msx repression of GnRH gene expression. Together, these results indicate that loss of

necdin could result in GnRH expression abnormalities and ultimately be the cause of the hypogonadotrophic hypogonadal phenotype present in PWS patients.

INTRODUCTION

Prader-Willi syndrome (PWS) is a multigenic neurodevelopmental disorder affecting approximately 1 in 15,000 live births (Goldstone, 2004). Diagnostic criteria include neonatal hypotonia, failure to thrive, hyperphagia, excessive weight gain, developmental delay, and hypogonadotropic hypogonadism (Gunay-Aygun et al., 2001), suggesting hypothalamic dysfunction. As a contiguous gene disorder, PWS is caused by loss of expression of genes in the 15q11-13 chromosomal region. Several maternally silenced, imprinted genes lie within this region, including *MKRN3*, *MAGEL2*, *NDN*, and *SNURF/SNRPN/IC*. Interestingly, the lack of single gene mutations causing PWS suggests that loss of multiple genes gives rise to this syndrome. However, it is thought that loss of expression of specific, individual genes contribute to distinct characteristics of the disorder. For this reason, it has been helpful to study the genes individually. Both necdin and Magel2 are members of the Melanoma Antigen Gene (MAGE) family. MAGE proteins are characterized by a conserved MAGE homology domain (MHD) which allows them to homo- and heterodimerize (Taniura et al., 2005). Along with Dlxin (also termed Maged1 or NRAGE), necdin and Magel2 are in the Type II MAGE subfamily and distinguished by their expression in normal, rather than mainly in tumorigenic tissues (Barker and Salehi, 2002) like their Type I counterparts.

Mouse chromosome region 7C is homologous to human 15q11-13; therefore, mutant mice are available as important models for the study of Prader-Willi syndrome.

Several genetic alterations created at 7C have been proposed as PWS models with varying phenotypes (Chamberlain et al., 2004; Ding et al., 2005; Gabriel et al., 1999; Kuwako et al., 2005; Skryabin et al., 2007; Stefan et al., 2005; Takazaki et al., 2002; Yang et al., 1998). Four different null-mutations in necdin have been created in mice (Gerard et al., 1999; Kuwako et al., 2005; Muscatelli et al., 2000; Ren et al., 2003; Takazaki et al., 2002; Tsai et al., 1999). Gerard *et al.* (Gerard et al., 1999), reported variable respiratory defects corresponding to the respiratory compromise seen in children with PWS. Muscatelli *et al.* (Muscatelli et al., 2000), observed various behavioral and hypothalamic abnormalities, including a reduction in hypothalamic neuron populations in the adult, including 25% fewer gonadotropin-releasing hormone (GnRH) positive neurons in the medial preoptic area. Kuwajima, *et al.* (Kuwajima et al., 2004) and Kuwako, *et al.* (Kuwako et al., 2005), reported reduced differentiation of GABAergic neurons and sensory neuron defects in their necdin-null mice. Additionally, mice null for Nhlh1 and 2, proteins known to activate necdin gene expression (Good et al., 1997; Kruger et al., 2004), have fertility defects and reduced numbers of GnRH neurons.

GnRH is the central regulator of the hypothalamic-pituitary-gonadal axis, and thus, GnRH neurons are critical for reproductive function. GnRH mRNA is first detected in the olfactory placode at embryonic day (e) 11.5 (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a). At e13.5, the neurons produce GnRH decapeptide and migrate across the nasal septum into the forebrain, where by e16 (Schwanzel-Fukuda et al., 1992; Schwanzel-Fukuda and Pfaff, 1989), most reach their proper

locations in the hypothalamus, allowing them to extend their axons to secrete GnRH into the hypophyseal portal system and execute their role in reproductive function. In this way, GnRH neuron migration, development, and gene expression all occur coincidentally and, may, in fact, be mechanistically related (Simonian and Herbison, 2001). There are only approximately 800 GnRH neurons dispersed in the adult mouse septohypothalamic region (Wray et al., 1989a), making analysis of their molecular mechanisms particularly difficult. Thus, we have utilized immortalized cell lines as *in vitro* models of GnRH neurons. GT1-7 cells display characteristics of mature GnRH neurons (Mellon et al., 1990; Silverman et al., 1992; Wetsel et al., 1992), producing high levels of GnRH in the characteristic pulsatile pattern. In contrast, Gn11 cell lines (Radovick et al., 1991) are migratory and secrete low levels of GnRH, consistent with an immature GnRH neuron.

We have previously shown that the Msx homeodomain family transcriptional repressor proteins regulate the GnRH gene and inhibit the development and migration of GnRH neurons (Givens et al., 2005). These actions are countered by Dlx homeodomain family transcriptional activators (Givens et al., 2005) binding to the same sequence recognition sites in the GnRH gene, though the mechanism responsible for regulating their differential binding and activities is unknown. Necdin and Dlxin-1, both members of the family of type II MAGE proteins, interact with Msx and Dlx proteins (Barker and Salehi, 2002; Masuda et al., 2001). However, these studies have all been performed with highly overexpressed binding partners, which leaves their physiological relevance in question. Specifically, in muscle cells, necdin and

Dlxin-1 relieve Msx repression of the Wnt1 promoter to promote muscle cell differentiation (Brunelli et al., 2004; Kuwajima et al., 2004).

The majority of Prader-Willi syndrome patients display defects in sexual development generally attributed to hypogonadotropic hypogonadism (Burman et al., 2001), potentially implicating the GnRH neuron or pituitary gonadotrope. Adult necdin null mice have reduced numbers of GnRH neurons (Muscatelli et al., 2000), suggesting that loss of necdin could affect fertility in PWS through actions on GnRH gene expression. Herein, we demonstrate that necdin activates GnRH gene expression and relieves Msx repression of GnRH transcription. We show that necdin is present in an endogenous protein complex with Msx1 in GT1-7 cells, and that Dlxin is likely a component of this complex with Msx and necdin. Therefore, we propose that the necdin interaction with Msx interferes with its repression of GnRH gene transcription, thereby playing an indirect role in activation of the GnRH gene. Since patients with PWS lack necdin expression, they may be unable to properly active GnRH and this could contribute to the etiology of infertility in Prader-Willi syndrome.

RESULTS

Necdin is Expressed in GT1-7 But Not Gn11 Cells

To identify candidate genes critical for GnRH neuron development, we performed an Affymetrix microarray screen comparing mRNA from Gn11 versus GT1-7 cells. Over 2000 transcripts showed significantly higher levels in GT1-7 versus Gn11 cells. In particular, necdin mRNA levels were 1000-fold higher in GT1-7 than Gn11 cells ($p= 2x10^{-61}$). Interestingly, other MAGE family members, Maged1 (encoding Dlxin-1 protein) and Magel2, as well as a Necdin regulating gene, Nhlh1 (NSCL-1) (Kruger et al., 2004), were not significantly different between the two cell lines (Figure 3-1A).

The differential expression of necdin between these two cell lines is also evident at the protein level. Western blots of whole cell protein extracts from GT1-7, Gn11, NIH3T3, and LβT2 (pituitary gonadotrope) cells show that GT1-7 cells express high levels of necdin protein, while it is undetectable in the other cell lines (Figure 3-1B). Thus, necdin is differentially expressed between immature and mature GnRH neuronal model cell lines, suggesting that it might be important for the development of these cells and expression of GnRH.

Necdin Activates GnRH Gene Expression and Relieves Msx Repression of GnRH Gene Expression

To determine whether necdin regulates GnRH gene expression, we transiently co-transfected Gn11 cells with a Necdin expression vector and rat GnRH-luciferase reporter plasmids. We utilized Gn11 cells due to their lack of endogenous necdin expression. GT1-7 cells express such high levels of necdin that co-transfection of a Necdin expression vector would not be expected to consistently and significantly increase levels reporter levels. Both a luciferase reporter containing the intact -5 kb upstream region of the rat GnRH gene and a reporter driven by the well-characterized rat GnRH regulatory elements: upstream enhancer, enhancer, and promoter (GnRHue/e/p) (Givens et al., 2004) were significantly induced by necdin (Figure 3-2A) over-expression. Therefore, necdin is sufficient to activate GnRH gene expression and the activation effect localizes to the known enhancer and promoter regions.

Since MAGE family members interact with, and regulate gene expression through, Msx and Dlx homeodomain proteins (Masuda et al., 2001), and Msx and Dlx proteins regulate GnRH transcription during development (Givens et al., 2005), we investigated the effect of necdin on Msx and Dlx regulation of the GnRH gene. As previously shown, over-expression of Msx1 significantly decreased GnRH transcription (Figure 3-2B) (Givens et al., 2005); interestingly, however, necdin prevents this repression by Msx1 (Figure 3-2B). In contrast, confirming previous

studies, Dlx proteins significantly increase GnRH reporter activity (Givens et al., 2005). However, co-expression of necdin did not alter the effects of Dlx on GnRH activity (Figure 3-2C), indicating that, in this case, necdin selectively acts through Msx homeodomain proteins. Additionally, overexpression of Magel2, another Prader-Willi candidate gene and MAGE family member, did not affect basal or necdin-activated GnRH gene expression, nor did the related MAGE protein, Dlxin-1 (Figure 3-3).

Necdin Activates GnRH through Msx/Dlx Binding Sites

If necdin is activating through prevention of Msx repression, the characterized Msx DNA-binding sites in the GnRH regulatory sequences should be necessary for activation by necdin. The GnRH enhancer and promoter each have two Q50 homeodomain binding elements at -1632 bp and -1619 bp and -53 bp and -38 bp, respectively, all of which bind Msx and Dlx family members (Givens et al., 2005). A GnRH reporter with mutations in all four characterized Msx/Dlx binding sites has previously been shown to eliminate the repressive effect of Msx (Givens et al., 2005). When necdin was transiently co-transfected into Gn11 cells with a GnRHue/e/p-luc reporter (Figure 3-4A) carrying these mutations, there was no significant activation by necdin (Figure 3-4B), demonstrating that the regulation by necdin requires the four known Msx-binding sites in the GnRH enhancer and promoter.

Necdin is Necessary for Proper GnRH Gene Expression

To determine whether necdin is required for GnRH gene expression, we examined the effect of knocking down necdin protein on GnRH expression in GT1-7 cells. siRNA duplex pools targeting necdin, Cyclophilin B, or a non-targeting control were transiently transfected into GT1-7 cells, which normally express high levels of endogenous necdin and GnRH. In Figure 3-5A, immunoblots of whole cell proteins show that knock down of necdin and Cyclophilin B proteins was specific to the appropriate siRNA duplex pool.

We next examined endogenous GnRH mRNA levels in GT1-7 cells in which necdin had been knocked down. RNA levels were analyzed from mock, non-targeting control, and necdin siRNA duplex pool transfected GT1-7 cells by quantitative RT-PCR (Q-PCR). GT1-7 cells subjected to siRNA knockdown of necdin expressed significantly less GnRH mRNA than did cells transfected with control siRNAs (Figure 3-5B). Thus, necdin is necessary for normal GnRH gene expression in GT1-7 cells.

To further establish that necdin knockdown affected GnRH expression at the transcriptional level, we co-transfected GT1-7 cells with siRNA duplex pools and either wild-type or mutant GnRH-luc reporters. Activity of the GnRHue/e/p-luc reporter was reduced 37% when necdin protein was knocked down (Figure 3-5C). Importantly, however, the mutant GnRHue/e/p-luc reporter showed no decrease in activity, confirming that regulation of GnRH by necdin requires the Msx/Dlx binding sites.

Necdin Interacts with Msx

Since necdin increases GnRH gene expression through Msx binding sites, we examined whether these proteins interact within the GT1-7 cells by co-immunoprecipitating endogenous necdin and Msx1 from GT1-7 cells. Msx1 was immunoprecipitated with an anti-necdin antibody, but not with the IgG control, demonstrating an interaction between endogenous necdin and Msx1 in GT1-7 cells (Figure 3-6A).

Another MAGE family member, Dlxin-1 (encoded by the Maged1 gene), has also been shown to form a protein complex with necdin and Msx/Dlx family members (Kuwajima et al., 2004). Msx1-FLAG and Dlxin-1-FLAG expression vectors were transfected into GT1-7 cells, then whole cell proteins were immunoprecipitated with anti-FLAG antibody. Western blots for necdin demonstrated that necdin interacts with both Msx1 and Dlxin-1 in GT1-7 cells (Figure 3-6B). Consequently, Dlxin-1 may also be present within the Msx and necdin protein complex that regulates GnRH gene expression.

We have previously shown that Groucho-related gene (Grg) family co-repressors can interact with Msx and to augment their repressor function in GnRH transcription (Rave-Harel et al., 2005). *In vitro* interaction assays mapped the interaction between these two proteins to the Q domain on the Grg4 protein and the N-terminal half of the Msx1 protein, which contains an engrailed homology domain (FxIxxIL) known to facilitate protein-protein interactions within other proteins

(Tolkunova et al., 1998). Additionally, other studies have shown that Grg co-repressors interact with engrailed homology domains within other homeodomain proteins (Jimenez et al., 1999; Lopez-Rios et al., 1999; Muhr et al., 2001). One mechanism by which necdin could be interfering with Msx repression of GnRH is by recruiting a co-repressor to Msx. Therefore, we examined whether proteins binding the engrailed homology domain (i.e. Grg) could be playing a role in necdin interference of Msx repression of GnRH. In order to do this, we utilized the Msx1-FLAG expression vector described above and generated a mutant version which has been shown to virtually abolish the interaction of Groucho to an engrailed domain (Tolkunova et al., 1998). When this mutant Msx1-FLAG (Msx1ΔEh) was overexpressed in GT1-7 in parallel with the wild-type Msx1-FLAG, necdin immunoprecipitation was not affected (Figure 3-6C), suggesting that the necdin-Msx protein complex does not require proteins that bind through the engrailed homology domain, such as Grg co-repressors.

Necdin Does Not Change the Cellular Localization of Msx

Another mechanism by which necdin could be relieving Msx repression of the GnRH gene, is to change its localization out of the nucleus, so that it can no longer bind DNA. MAGE proteins have been observed to change localization to the nucleus or cytoplasm depending on the presence or activation state of other proteins within the cell (Matsuda et al., 2003). We utilized Gn11 cells, as their lack of endogenous necdin

allowed us to control its expression and detect Msx1-FLAG in cells with or without necdin co-expression. Without necdin expression, Msx1-FLAG is detected only within the nucleus of the Gn11 cells (Figure 3-7A). When necdin is co-transfected into the Gn11 cells, Msx1-FLAG localization remains in the nucleus (Figure 3-7B), suggesting that the presence of necdin does not move Msx1 out of the nucleus, thus, allowing inactivation Msx1 repression of GnRH gene expression.

DISCUSSION

Hypogonadism is one of the major diagnostic criteria for Prader-Willi syndrome (Gunay-Aygun et al., 2001). It is usually hypogonadotrophic and at least partially attributed to hypothalamic dysfunction (Eiholzer et al., 2006). As such, the infertility generally associated with Prader-Willi syndrome is an important topic of investigation. Several genes that reside within the human 15q11-13 region are inactivated in Prader-Willi syndrome, including two MAGE family genes, *NDN* and *MAGEL2*. Hypothalamic insufficiency has been suggested in necdin-null mice (Muscatelli et al., 2000), making necdin an interesting candidate for contribution to the hypogonadal phenotype. The results presented herein are the first demonstrating necdin regulation of GnRH gene expression.

The microarray comparison of mRNAs in immature Gn11 versus mature GT1-7 GnRH cell lines revealed that necdin was the most differentially expressed transcript, suggesting a critical role for necdin in the maturation of GnRH neurons. Necdin is a marker of mature CNS neurons and is thought to play a role in neuronal differentiation (Andrieu et al., 2003; Yoshikawa, 2000), consistent with its high expression in GT1-7 and absence from Gn11 cells. In fact, transfection of necdin into immature Gn11 cells resulted in activation of GnRH transcription. Since necdin is known to exist in protein complexes with Msx repressor and Dlx activator homeodomain proteins in the regulation of the Wnt1 promoter in muscle cells (Barker and Salehi, 2002; Masuda et al., 2001) and we had previously shown that Msx

repression and Dlx activation contribute to differential regulation of GnRH gene expression during developmental migration (Givens et al., 2005), we addressed the interaction of necdin with Msx and Dlx in GnRH gene expression. Necdin activation of GnRH transcription was dependent upon the four known Msx/Dlx binding sites, yet we were unable to detect necdin binding to these elements, either by EMSA supershift with necdin antibodies or using in vitro synthesized necdin (data not shown). siRNA knockdown of necdin in GT1-7 cells reduced both endogenous GnRH mRNA transcripts and GnRH reporter gene transcription and this effect was also dependent upon the four Msx/Dlx binding elements. Necdin relieves Msx repression of GnRH gene transcription, though Dlx activation of GnRH was not affected, indicating that necdin is specifically preventing Msx repression through the GnRH enhancer and promoter binding sites. In fact, Msx1 co-immunoprecipitates from GT1-7 cells with necdin, demonstrating that a protein complex containing the endogenous Msx1 and necdin proteins exists in these GnRH neurons. Another MAGE family member, Dlxin-1, also has been shown to complex with Msx and Dlx, in the presence of necdin (Barker and Salehi, 2002; Masuda et al., 2001), and Dlxin-1-FLAG transfected into GT1-7 cells did co-immunoprecipitate with necdin. However, Dlxin-1 did not affect basal or necdin-activated GnRH gene expression, nor did the related protein, Magel2. These studies indicate that necdin acts in a complex with Msx1 to interfere with Msx repression of GnRH transcription, while Magel2 and/or Dlxin-1 are either not necessary or their endogenous levels are sufficient for the action of the Msx/necdin complex.

Necdin interference with Msx repression of GnRH gene expression appears to occur through formation of an Msx/necdin protein complex and requires the Msx-binding elements in the GnRH gene. This interaction with necdin might prevent Msx repression by interfering with Msx DNA binding, could affect Msx recruitment of a co-repressor, may have an effect on Msx protein stability, or perhaps its localization to the nucleus. Though Grg family co-repressors have been shown to interact with Msx homeodomain proteins and to augment their repressor function in GnRH transcription (Rave-Harel et al., 2005), mutating the engrailed homology domain by which Grg and Msx proteins interact, does not affect the interaction of necdin and Msx1 and Grg repression of GnRH gene expression was not affected by necdin (data not shown). However, Grg proteins also act as co-repressors for Oct-1, which acts through other binding sites in the GnRH gene, and may well be their primary target in GnRH neurons. In addition, Msx proteins were not reduced in level or detected outside of the nucleus in Gn11 cells transfected with necdin, indicating that necdin may not act by altering the localization or stability of Msx proteins.

In summary, we have identified the MAGE protein, necdin, as a key regulator of GnRH gene expression. Necdin is inactivated in Prader-Willi syndrome and individuals with PWS patients are typically infertile. Necdin activates GnRH gene transcription and is capable of relieving the characterized Msx repression of the GnRH gene. This is confirmed by the demonstration that necdin activation of GnRH is occurring through the Msx binding sites in the GnRH regulatory regions. We also show that in addition to being sufficient for activation of the GnRH gene, necdin is

necessary for proper GnRH expression. Within a GnRH model cell line, necdin exists in a protein complex including Msx (and possibly Dlxin) which may contribute to its molecular function. Ultimately, a lack of necdin results in a reduction of GnRH gene expression, which may contribute to infertility in Prader-Willi syndrome.

MATERIALS AND METHODS

Cell Culture and Transfections. GT1-7, Gn11, NIH3T3, and LβT2 cells were cultured in Dulbecco's modified Eagle's medium with 4.5% glucose, 10% fetal bovine serum and 1x penicillin-streptomycin in 5% CO_2 at 37°C. GT1-7 and Gn11 cells were split into 24-well plates for transfection at 90,000 and 50,000 cells per well, respectively. 400 ng/well of rat GnRHue/e/p-luciferase or mutant rat GnRHue/e/p-luc reporter was co-transfected with 100 ng/well of pHismaxC-Necdin, pCB6+Msx1, pCAGG-Dlx1, pCB6+Dlx2, or pcDNA3Dlx5, along with thymidine kinase-β-galactosidase as an internal control. Appropriate empty vectors were utilized as controls. Transfections used FuGENE 6 reagent (Roche) according to manufacturer's protocols. The mutant rat GnRHue/e/p reporter contains mutations previously described (Givens et al., 2005) generated in the rat GnRHue/e/p-luc vector by targeted mutagenesis (Stratagene Site-Directed Mutagenesis kit).

Cells for reporter assays were harvested 48 hours after transfection in lysis buffer (100 mM potassium phosphate and 0.2% Triton-X-100, pH7.8) unless otherwise noted. Luciferase assays were performed as previously described (Givens et al., 2005) and β-galactosidase assays were performed as directed by the manufacturer (Tropix, Bedford, MA). Luciferase values were normalized to β-galactosidase values as internal control, to control for transfection efficiency. Values were always compared to empty vector control. Experiments were performed in quadruplicate and repeated a minimum of three times. Data represent the mean, ± SEM of at least three

independent experiments, each performed in quadruplicate. Statistical analyses are described in their appropriate figure legends.

RNA Isolation and Microarray. Total RNA extraction was carried out using Ultraspec™ RNA Isolation System (Biotecx Laboratories Inc Houston, TX), according to manufacturer's instructions, from GT1-7 and Gn11 cells grown in monolayer on 10 cm plates. Total RNA was resuspended in DEPC H_20 and reprecipitated with 3 M NaOAC, pH 5.2 and then ethanol to eliminate any residual phenol or guanidinium salts. RNA was submitted to the UCSD-VA GeneChip Core facility for analysis using Affymetrix MOE430A microarrays. Duplicate assays were performed for each with independently isolated RNA from independent batches of cells. Data was analyzed using GeneSpring (Silicon Genetics) software. Additional analysis was performed using VAMPIRE (Hsiao et al., 2004). We have previously shown that the statistical approach implemented in VAMPIRE finds the majority of genes found by standard ANOVA approaches as well as many genes that are missed by the other methods (Lawson et al., 2007; Zhang et al., 2006). VAMPIRE is also more robust than ANOVA-based procedures at low sample number.

Protein Extract Preparation and Co-Immunoprecipitation. Whole cell extract was prepared from cells by washing twice with 1X PBS, followed by lysis (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 1x protease inhibitor cocktail (Sigma), and 1 mM PMSF, pH 7.4) directly on tissue culture dishes. Cell lysate mix

was allowed to incubate while rocking at 4°C for 15 minutes then centrifuged for 10 minutes to clear debris. Crude protein was either used immediately or frozen at -80°C for future use. 20 µg protein was loaded for SDS-PAGE analysis on 12% polyacrylamide mini-gels.

Two co-immunoprecipitation methods were used, magnetic Protein A beads and EZ-View Red FLAG Affinity gel. For the magnetic co-immunoprecipitation, pre-cleared 200 µg GT1-7 whole cell lysate was incubated with 4 µg either anti-necdin (Abcam ab18554) or anti-rabbit IgG (Santa Cruz sc-2027) at 4°C for 1 hour. 25 µl Protein A Magnetic Beads (NEB) was added and incubated overnight at 4°C while rocking. Bead/protein complexes were washed 5 times, eluted in 2x SDS sample buffer at 70°C for 5 minutes. Alternatively, co-immunoprecipitation was performed using the EZ-View Red FLAG Affinity Gel (Sigma) per manufacturer's instructions. 250 µg GT1-7 whole cell extract over-expressing FLAG-Msx1 or FLAG-Dlxin1 was used. 4% of protein was reserved for visualizing input. Immunoprecipitated proteins were dissociated from beads by boiling for 5 minutes in 2x SDS sample buffer. For either method, precipitate was split into two and loaded into 12% SDS polyacrylamide gels for protein separation. Proteins were transferred to PVDF and blocked overnight in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Anti-Msx1 at 1:1600 (Aviva ARP37094) or anti-necdin (Abcam ab18554) at 1:1000 or was diluted in blocking buffer. Anti-rabbit HRP secondary (1:5,000) (Amersham NA934V) was diluted in TBS-T wash buffer. Chemiluminescence was detected (Pierce SuperSignal West Pico) and exposed to film.

siRNA Knock-down and Quantitative RT-PCR. Necdin, non-targeting control, and cyclophilin B siRNA duplex pools (Dharmacon) were transfected into GT1-7 cells using DharmaFect3 reagent (Dharmacon). For protein knock-down analysis, immunoblots were performed on whole cell protein extracted 72 h after transfection as described above with the addition of an anti-cyclophilin antibody used at 1:1000 (Abcam ab16045).

Total RNA was harvested at 48 h post-transfection using the RNeasy mini kit (QIAGEN). cDNA was generated using 2 µg RNA and the SuperScript III First-Strand Synthesis System (Invitrogen). cDNA was diluted 1:10 to yield an equivalent of 50 ng starting RNA per reaction. All quantitative PCR reactions were performed in a 25 µl reaction using either iQ SYBR Green Supermix (BioRad) or Absolute Blue SYBR Green Fluorescein Mix (Thermo Scientific). The following primers were used: cyclophilin B forward: CGTGGCCAACGATAAGAAGA, cyclophilin B reverse: GAAGTCTCCACCCTGGATCA, GnRH forward: TGCTGACTGTGTGTTTGGAAGGCT, GnRH reverse: TTTGATCCACCTCCTTGCGACTCA under the following cycling conditions: 15 minutes at 95°C for enzyme activation, followed by 40 cycles of 95° for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Additionally, an 81 cycle step at 55°C for 30 seconds was performed at the end of the PCR cycling for melting temperature analysis to confirm purity. Standard curves for each product were generated using serial dilutions of known quantities of plasmid containing the appropriate cDNA within the same run as the samples. All quantitative PCR reactions were performed in

triplicate and repeated four times. Data was collected using the BioRad iQ5 detection system and relative quantities for GnRH were extrapolated from the standard curve based on threshold cycle (Ct) values. Replicates were averaged and divided by the mean of cyclophilin B within the same sample.

Co-transfections were performed as described above except siRNA duplex pools for a non-targeting negative control or Necdin were co-transfected with the rGnRH-luc and thymidine kinase β-galactosidase reporter plasmids using the DharmaFect 3 reagent. Assays and analysis were the same, except values were normalized to a mock-siRNA treated, co-transfected control.

Immunocytochemistry. Gn11 cells were cultured as above in 2-well Lab-Tek II Chamber Slides (Fisher). Cells were fixed in 3.7% formaldehyde for 10 minutes, permeabilized for 20 minutes in 0.2% NP40, 1% BSA, 10% goat serum in PBS, avidin blocked for 30 minutes in 20% goat serum, 5% BSA, avidin solution from Avidin/Biotin blocking kit per manufacturer's instructions (Vector Labs) in PBS, biotin blocked for 30 minutes according to manufacturer's instructions (Vector Labs), and incubated overnight at 4°C in anti-necdin (Abcam ab18554, 1:1000) and anti-FLAG Cy3 (Sigma, 1:110) antibody diluted in blocking solution (20% goat serum, 5% BSA). Cells were incubated for 30 minutes with biotinylated goat-anti-rabbit secondary antibody (Molecular Probes) used at 1:500 in blocking solution. Strepavidin-Alexa488 fluorescent conjugate (1:200 in PBS) in was incubated with cells for 1 hour in the dark. Slides were mounted with coverslips using VectaShield

Hard Set Mounting Media with DAPI (Vector Labs). All incubators were done at room temperature unless otherwise noted. Each antibody individually and no primary antibody controls were also performed in parallel. Fluorescence was visualized with a Nikon Eclipse TE2000-U microscope.

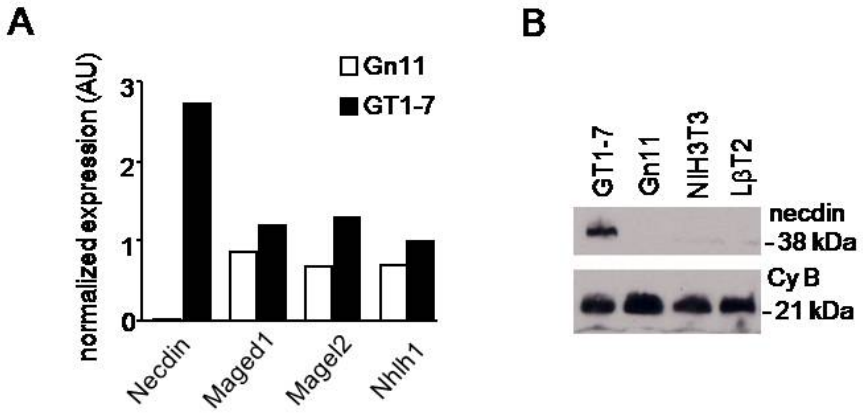

Figure 3-1. Necdin is expressed in mature GnRH neurons. A) Affymetrix microarray chip MOE430A was used to compare total RNA from GT1-7 cells versus Gn11 cells. Data was analyzed by GeneSpring software. The mean of experiments is shown. B) Necdin and cyclophilin B (Cy B) protein expression in GT1-7, Gn11, NIH3T3, LβT2 whole cell protein.

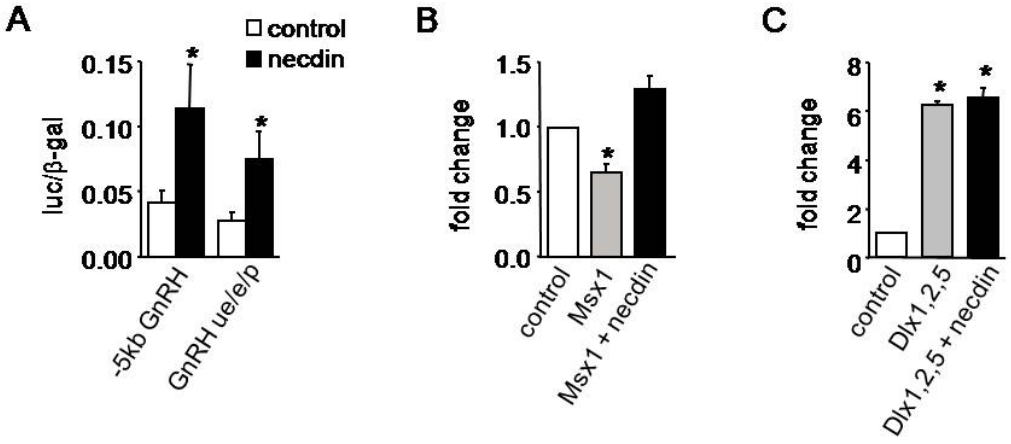

Figure 3-2. Necdin regulates GnRH gene expression. A) Either -5 kb rat GnRH-luc or rat GnRHue/e/p-luc was co-transfected with necdin or empty vector into Gn11 cells. B) The GnRHue/e/p-luc reporter was co-transfected with either Msx1 or Msx1 and necdin. C) The GnRHue/e/p-luc reporter was co-transfected with Dlx1, Dlx2, and Dlx5, with or without necdin. Where indicated, values shown represent means of luciferase/β-galactosidase normalized to empty reporter vector pGL3. Means were compared by one-way ANOVA and Tukey-Kramer HSD and * indicates ($p<0.05$).

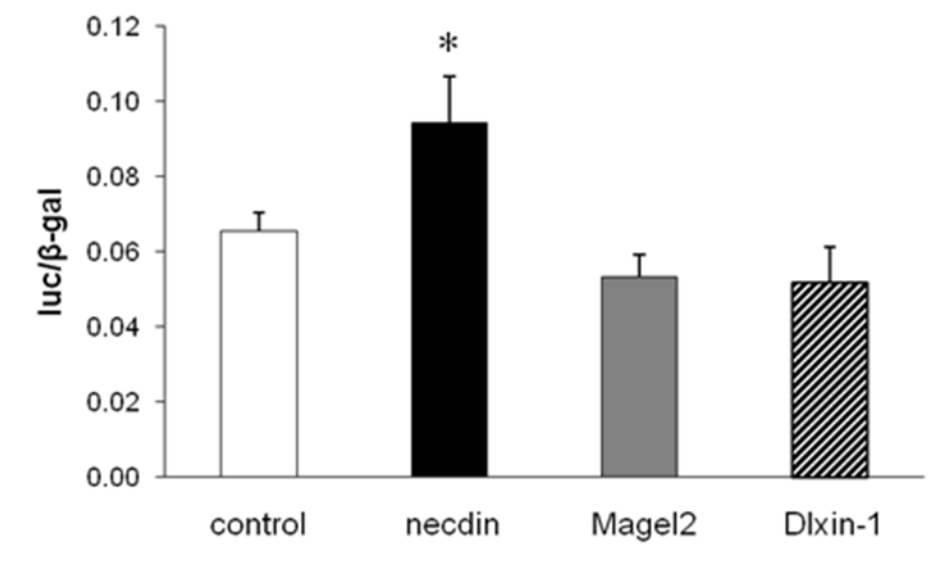

Figure 3-3. The other two closely related MAGE family members do not contribute to GnRH gene expression. Gn11 cells were co-transfected with rat GnRHue/e/p-luciferase reporter in addition to either control empty vector, necdin expression vector, Magel2 expression vector, or Dlxin expression vector. Values were normalized to thymidine kinase-β-galactosidase internal control. Error bars represent the SEM. Means were compared by one-way ANOVA and Tukey-Kramer HSD and * indicates ($p<0.05$).

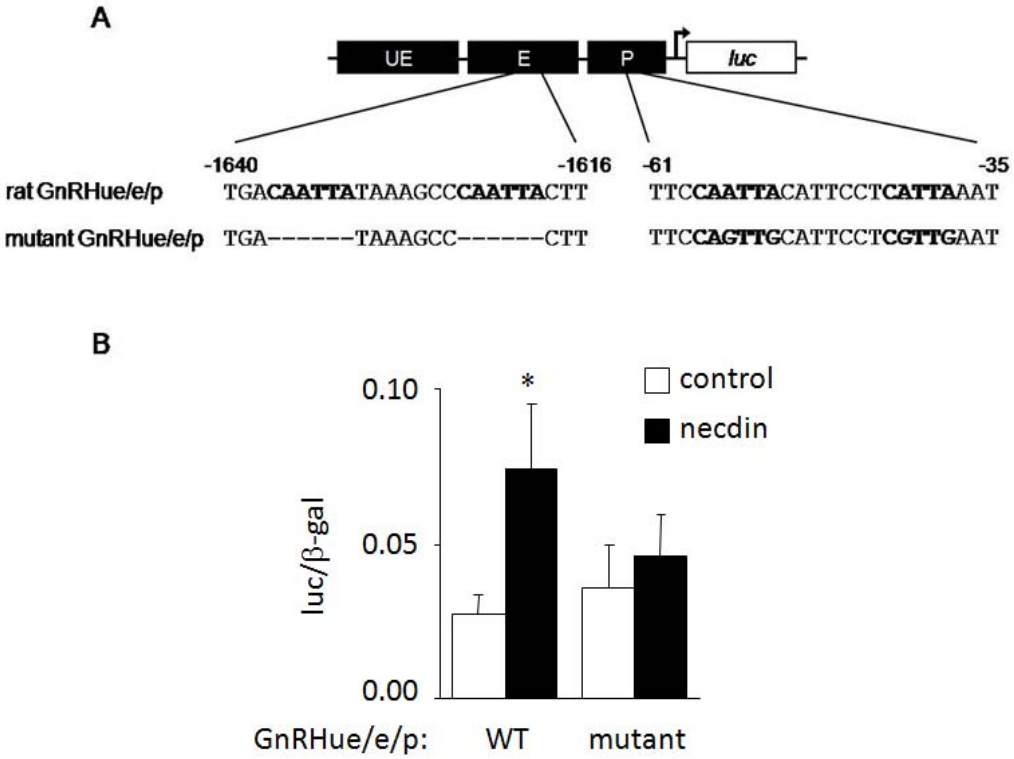

Figure 3-4. Necdin activation of GnRH requires Msx/Dlx binding sites. A) The rat GnRH-luc reporter contains four Msx/Dlx binding sites at -1632 bp, -1619 bp, -53 bp, and -38 bp relative to the transcription start site. The top sequence is the wild type rat GnRH; the bottom sequence contains mutations in the four Msx/Dlx sites in the GnRH promoter and enhancer. B) Necdin expression vector was co-transfected into Gn11 cells along with either GnRHue/e/p or mutant GnRHue/e/p luciferase reporters. Means were compared by Student's T-test and * indicates significant values ($p<0.05$).

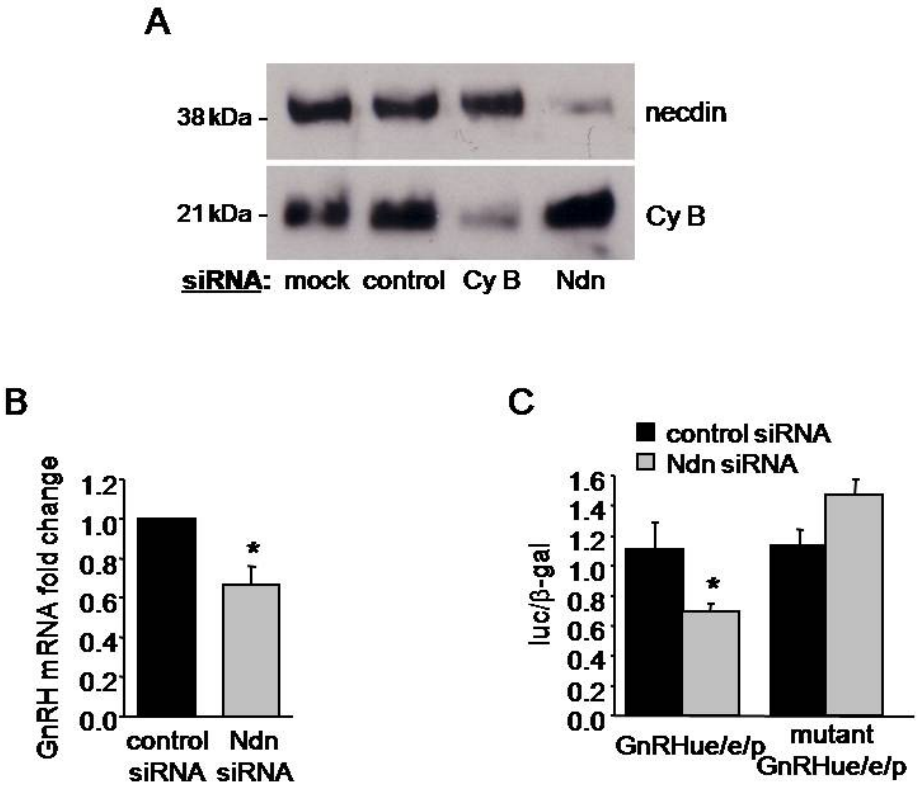

Figure 3-5. Necdin is necessary for GnRH gene expression. Knockdown of necdin in GT1-7 cells results in a decrease in GnRH mRNA and gene expression. GT1-7 cells were either mock-transfected or transfected with non-targeting siRNA duplexes (control), cyclophilin B (Cy B) siRNA duplexes (as a positive control), or necdin (Ndn) siRNA duplexes (Dharmacon). A) Specific knockdown of Ndn and Cy B was detected by western blot of whole cell lysates harvested at 48 hours. B) GT1-7 cells were transfected with either control or Ndn siRNA duplexes. RNA was harvested at 72 hours and reverse transcribed. GnRH was measured by quantitative RT-PCR and data are shown as fold difference in expression compared to non-targeting siRNA negative control. Values represent the means of four independent experiments and ***** indicates values significantly different as determined by Student's T-test ($p<0.05$). C) GT1-7 cells were mock or co-transfected with control or Ndn siRNA duplexes and either GnRHue/e/p-luc or mutant GnRHue/e/p-luc reporters. Data shown are normalized to mock siRNA transfection. Means were compared by one-way ANOVA and Tukey-Kramer HSD and * indicates ($p<0.05$).

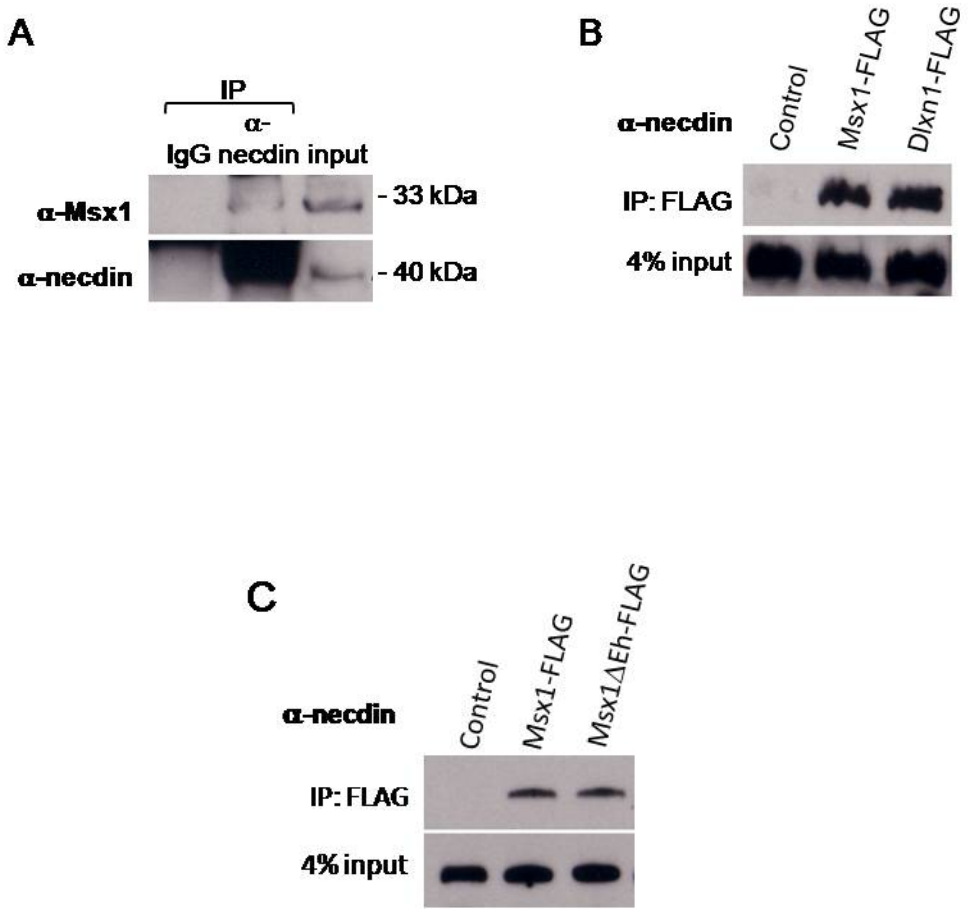

Figure 3-6. Necdin interacts with Msx1. A) Whole cell lysate from GT1-7 cells was immunoprecipitated with either anti-necdin antibody or rabbit IgG. Msx1 and necdin were detected by western blotting the immunoprecipitated proteins. 10% input was run for Msx1 and 2% for necdin. B) GT1-7 cells were transiently transfected with Msx1-FLAG or Dlxin1-FLAG expression vector or empty vector control. Whole cell extract was immunoprecipitated with anti-FLAG M2 Affinity Gel. IP reactions and 4% input were resolved by SDS-PAGE and necdin detected by immunoblot. C) GT1-7 cells were transiently transfected with either Msx1-FLAG or a mutant Msx1-FLAG (Msx1ΔEh-FLAG) or empty vector control. Immunopreciptiation and necdin detection were as above in (B).

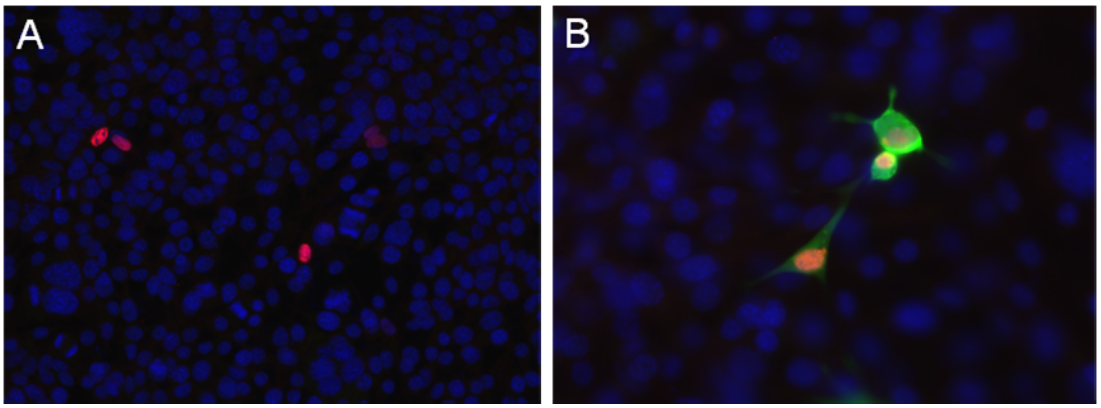

Figure 3-7. Necdin expression does not change the cellular localization of Msx. A) Msx1-FLAG over-expression in Gn11 was detected with an anti-FLAG-Cy3 antibody conjugate (red). Image shown is at 20x magnification. B) Gn11 cells were transiently transfected with both necdin and Msx1-FLAG expression vectors and then subjected to immunofluorescence using the anti-FLAG-Cy3 antibody (red) or anti-necdin (green). Image shown is at 40x magnification. Blue staining is DAPI nuclear labeling.

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CHAPTER IV

The Role of the Prader-Willi Candidate Genes, Necdin and Magel2 in the Development of Gonadotropin-Releasing Hormone Neurons

Necdin and Magel2 are two related proteins that are inactivated in Prader-Willi syndrome (PWS). PWS is a complex neurogenetic disorder with major diagnostic criteria including hyperphagia induced obesity and hypogonadotrophic hypogonadism. *NDN* and *MAGEL2* are two of only four protein encoding genes and map to the Prader-Willi syndrome critical region of human chromosome 15q11-13 and are syntenic to mouse chromosome 7C. Adult mice lacking necdin expression were observed to have reduced GnRH neuron numbers but the role of necdin in the GnRH neuron is unknown. Herein, we show that developing necdin-null mice have significantly reduced GnRH numbers along their migratory path. At embryonic day 13, early in GnRH neuron development, this phenotype is more severe with very few GnRH neurons crossing the cribriform plate and in the hypothalamus. By embryonic day 17, when the GnRH neuronal population should be well-established, necdin-null mice have significantly reduced GnRH neuron numbers in the brain, however, overall, the number of GnRH neurons is no longer significantly different between wild-type. Furthermore, we demonstrate that this is specific to necdin, as Magel2-null mice do not have fewer than normal GnRH neurons. Overall, these results suggest an

important role for necdin in GnRH neuron development and suggest that this may contribute to the hypogonadotrophic hypogonadism observed in Prader-Willi patients.

INTRODUCTION

Loss of expression of genes that lie within the human 15q11-13 chromosomal region is responsible for the complex neurogenetic disorder, Prader-Willi syndrome (PWS). It is estimated to affect 1 in 10,000 to 20,000 individuals (Bittel and Butler, 2005). Major diagnostic criteria for PWS include infantile hypotonia leading to failure to thrive, subsequent hyperphagia leading to central obesity, developmental delay, hypogonadism, and 15q11-13 genetic abnormality (Goldstone, 2004). Additionally, neuroendocrine disturbances in many cases (Burman et al., 2001) indicate hypothalamic abnormalities.

Three main genetic forms of Prader-Willi syndrome have been discovered to date. Paternal deletion makes up approximately 75% of the known cases of PWS. This category can be further divided into two categories, one containing a larger deletion and of which accounts for 40%, with the remaining 60% of cases containing a smaller deletion (Bittel and Butler, 2005). About 22% of the cases reported have maternal uniparental disomy. Since most of the genes within the PWS locus are maternally-silenced by imprinting, this results in loss of expression of these genes. The remaining PWS cases (<5%) are the result of imprinting defects, and are responsible for the heritable occurrences. This generally occurs as a result of a mutation in the imprinting center (IC) which also lies in the chromosome 15 region. Consequently, the grandmaternal imprinting of is not erased and the paternal copy is improperly silenced, resulting in loss of expression.

Several paternally-expressed, maternally-imprinted genes are found in the 15q11-13 locus, including *MKRN*, *MAGEL2*, *NDN*, and *SNURF/SNRPN*. While single gene mutations have not been reported to result in Prader-Willi syndrome (Nicholls and Knepper, 2001), it is thought that loss of individual genes contributes to the highly variable and distinct phenotypes associated with PWS. It is for this reason, that many researchers have explored these genes individually. Interestingly, both *MAGEL2* and *NDN* give rise to proteins (Magel2 and necdin, respectively) that are members of the melanoma antigen gene (MAGE) family and have been implicated in several cellular processes.

The Prader-Willi locus on human chromosome 15q11-13 is highly homologous to mouse chromosomal region 7C. Therefore, mouse genetics within this region can provide important insight into PWS. Several mouse models for PWS have been reported. However, no one mutation gives rise to the complete array of Prader-Willi characteristics. Mutations generating large deletions (Gabriel et al., 1999; Stefan et al., 2005) or creating IC defects (Chamberlain et al., 2004; Yang et al., 1998) result in a number of PW-like symptoms, including hypotonia, failure to thrive, and growth deficiency. Individual gene mutations have also produced specific Prader-Willi characteristics. Loss of *Magel2* expression gives rise to circadian rhythmicity abnormalities (Kozlov et al., 2007) and growth defects (Bischof et al., 2007). Mutation in *Ndn* result in neonatal respiratory distress (Gerard et al., 1999), neuronal defects (Kuwajima et al., 2006; Kuwako et al., 2005), behavioral alterations (Muscatelli et al., 2000), and a reduction in hypothalamic neuronal populations

(Muscatelli et al., 2000). Interestingly, no mouse models for PWS have been reported to have an obesity or obvious infertility phenotype. When necdin activating proteins, Nhlh1 and/or Nhlh2 are knocked out in the mouse, fertility defects and obesity were observed (Cogliati et al., 2007; Good et al., 1997; Kruger et al., 2004), although this may be an indirect effect. However, the *Ndn* null mice reported by Muscatelli *et al* (Muscatelli et al., 2000) did have ~25% fewer gonadotropin-releasing hormone (GnRH) neurons in the adult medial preoptic area and *Nhlh* null mice also had reduced GnRH neuron numbers(Kruger et al., 2004), indicating necdin may be important for hypothalamic function.

GnRH neurons (and the GnRH neuropeptide they secrete) are fundamental to the regulation of the hypothalamic-pituitary-gonadal (HPG) axis and are essential for reproductive function. GnRH neurons are thought to originate in the vomeronasal organ (VNO), outside the CNS, at approximately embryonic day (e) 11 (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a) when the GnRH decapeptide can be detected. These neurons then migrate across the cribriform plate and into the hypothalamus, where they extend axons that will eventually release GnRH into the hypophyseal portal system and carry out their function in the HPG axis.

One of the major diagnostic criteria for Prader-Willi syndrome is hypogonadism and generally reported as hypogonadotrophic (Burman et al., 2001). While this implies a defect in the hypothalamic or pituitary components of the HPG axis, this has yet to be specifically analyzed. Mice with genetic mutations within chromosome 7 analogous to the human PWS region in chromosome 15q11-13, allow

for analysis of the contribution of individual genetic components to specific physiological aspects of Prader-Willi syndrome. The genetic element that plays a role in the hypogonadism and fertiltiy of PWS has not been characterized, however, Muscatelli *et al* (Muscatelli et al., 2000), observed reduced GnRH neuron numbers in necdin null mice, indicating a role for necdin in GnRH neuron development. Herein, we demonstrate the necdin is critical during GnRH neuron development and migration in the embryo. We describe a comprehensive analysis of necdin null GnRH neurons during their migratory route at both e13.5 and e17.5. We also show that this is specific to necdin as Magel2, a related MAGE family member and adjacent PWS candidate gene, does not display the same phenotypic characteristics. Therefore, we propose that necdin plays an important role in the development of GnRH neurons, and that loss of necdin is likely to be a factor in the hypogonadotrophic hypogonadism and infertility reported in Prader-Willi syndrome.

RESULTS

Necdin Null Mice have Fewer GnRH Neurons at e13.5

Adult necdin-null mice exhibit reduced numbers of GnRH neurons in the medial preoptic area (Muscatelli et al., 2000). However, the role of necdin during GnRH neuronal development has not been analyzed. GnRH immunohistochemistry was performed on sagittal sections of necdin-null embryos and their wild-type littermates at different stages of GnRH neuron development, and the total numbers of GnRH-positive cells were counted (Figure 4-1A-D). By e13.5, the full complement of GnRH neurons should be established predominantly in the nasal regions (Schwanzel-Fukuda and Pfaff, 1989). Wild-type e13.5 embryos had 1053 ± 83 GnRH positive cells in total, consistent with previous findings (Givens et al., 2005) (Figure 4-1C). In contrast, necdin-null littermates had significantly reduced numbers of GnRH neurons (563 ± 102). The pathway for GnRH neuron migration was divided into three areas: nasal (N), cribriform plate (CP), brain (B) (depicted in Figure 4-1A) to analyze progression of migration. Wild-type and necdin-null e13.5 mice had similar numbers of GnRH neurons in the nasal region, 351 ± 52 and 361 ± 24, respectively. However, both the cribriform plate and brain regions had significantly reduced numbers of GnRH-positive cells in necdin-null animals (CP: WT 379 ± 61 versus null 161 ± 33 and B: 241 ± 31WT versus 43 ± 29 null; Figure 4-1D). Thus, e13.5 necdin-null mice have significantly fewer neurons than wild-type. Specifically, fewer GnRH neurons

appeared to cross the cribriform plate into the brain, indicating that necdin plays a vital role in the developmental progression of GnRH neurons.

Necdin Null Mice Have Fewer GnRH Neurons in the Brain at e17.5

We then examined the number and location of GnRH positive neurons in necdin-null and wild-type e17.5 embryos (Figure 4-2A-D). Interestingly, by this point in development, the total number of GnRH neurons was comparable in the necdin-null mice and their wild-type littermates (wild-type 1175 ± 309 and necdin-null 1045 ± 77; Figure 4-2C). However, the number of GnRH-positive neurons located in the brain was still significantly lower in necdin-null animals (520 ± 27) compared to wild-type littermate embryos (758 ± 120; Figure 4-2D). While the numbers of GnRH neurons in the cribriform plate region were the same at e17.5, regardless of necdin expression, the number of GnRH expressing cells in the nasal region was higher in the necdin-null mice, however this was not statistically significant ($p=0.08$) by our analysis. These data indicate that the loss of necdin causes a more dramatic effect in early GnRH neuronal migration (46% decrease at e13.5) than in the later stage (e17.5). However, even at e17.5, the ~31% reduction in GnRH neurons in the brain is consistent with the ~25% reduction previously noted in the adult medial preoptic area (Muscatelli et al., 2000). Together these studies show that necdin regulates development of GnRH neurons.

Magel2 Null Mice Have Normal GnRH Neuron Numbers

Magel2 is also a type II MAGE family member and the gene encoding it lies just upstream of Necdin within the imprinted Prader-Willi chromosomal region on both the mouse and human chromosomes, making it an additional candidate for study. Mice lacking Magel2 expression (Bischof et al., 2007; Kozlov et al., 2007) were analyzed during GnRH neuron development. GnRH-expressing neurons were counted in developing Magel2-null mice at e13.5 (Figure 4-3) and their wild-type littermates. However, the numbers of GnRH-positive neurons was not different between wild-type mice (1063 ± 60) and mice lacking expression of Magel2 (1013 ± 103) (Figure 4-3A). Also shown are the GnRH neuronal counts in the specific nasal, cribriform plate, and brain regions as described above (Figure 4-3B). These numbers were also not observed to be significantly different between wild-type and null (438 ± 76 vs 350 ± 69, 361 ± 35 vs 392 ± 61, and 263 ± 56 vs 270 ±57, for the nasal, cribriform plate, or brain regions, respectively. This suggests that the GnRH neuronal phenotype demonstrated above is specific to necdin-null mice.

DISCUSSION

GnRH transcriptional activity is thought to be dependent on the developmental stage of the embryo (Simonian and Herbison, 2001), increasing as the neurons mature. We previously showed in Chapter III that necdin contributes to proper GnRH gene expression. We also know that specific expression of GnRH is controlled at the level of transcription (Pape et al., 1999; Skynner et al., 1999b; Suter et al., 2000). If necdin is truly playing a role in GnRH transcription, then this should be evident *in vivo*, and detectable by examining GnRH neurons in mice lacking necdin during their development. In support of this hypothesis, necdin appears important for GnRH neuron maturation *in vivo* as the neurons migrate into the brain. At e13.5, total GnRH neurons were 46% fewer than in their wild-type littermates, though the numbers of GnRH neurons remained the same in the nasal area of the necdin null embryos. Approximately 58% fewer GnRH-positive neurons were observed in the cribriform plate and 72% fewer entering the brain. By e17.5, total numbers were no longer different, however, there were still approximately 31% fewer GnRH neurons in the brain. This is consistent with the report that adult necdin-null mice have reduced numbers of GnRH neurons in the medial preoptic area (Muscatelli et al., 2000). Thus, the lack of necdin in the null animals affects GnRH neurons only after they have reached the cribriform plate and primarily affects those having reached the brain. GnRH neurons are thought to begin expressing necdin mRNA at e12 (Andrieu et al., 2003), as they are migrating toward the cribriform plate. Thus, our findings suggest

that the lack of necdin results in lower levels of GnRH transcription in the GnRH neuron and perhaps in a delay in GnRH neuronal migration or maturation resulting in fewer GnRH neurons ultimately reaching the hypothalamus. In this way, necdin could be involved in ensuring that GnRH gene expression is activated within a specific temporal and spatial window, allowing the GnRH neuron to mature and reach its target in the hypothalamus, where it can play its essential role in reproduction.

Several genes lie in the Prader-Willi candidate region, including one that encodes another type II MAGE family member, Magel2. The *Magel2* gene lies just 5' to *Ndn*, and their respective proteins can also interact *in vitro* (Kuwako et al., 2004). Interestingly, Magel2-null mice did not have reduced numbers of GnRH neurons when compared to wild-type, indicating that the effect on GnRH is specific to necdin. Our evidence supports the hypothesis in which a contiguous gene disorder such as Prader-Willi syndrome results in such varied characteristics as a result of the loss of expression of individual, specific genes.

Though Necdin null mice have not been reported to have obvious infertility, in mice, a reduction by as much as 66% of GnRH neurons has been demonstrated to still result in fertile females (Herbison et al., 2008). Thus, lack of necdin in patients affected by Prader-Willi syndrome could result in infertility, 1) if the reduction in GnRH neurons were more dramatic in humans than mice, 2) if a 30% reduction were sufficient for infertility in humans, or 3) if additional gene deficiencies in the Prader-Willi interval augment the reduction of GnRH neurons. Additionally, as many cases of hypogonadotropic hypogonadism have been categorized as idiopathic and the gene

defect has only been identified in 30% of patients (Pitteloud et al., 2007), it will be important to determine whether non-Prader-Willi syndrome patients with idiopathic hypogonadotropic hypogonadism exhibit mutations in the necdin gene.

In summary, we have identified the MAGE protein, necdin, as a key regulator of GnRH *in vivo.* Necdin is inactivated in Prader-Willi syndrome and individuals with Prader-Willi patients are typically infertile. Thus, lack of necdin results in decreased numbers of GnRH neurons, an action that may contribute to infertility in Prader-Willi syndrome.

MATERIALS AND METHODS

Histology and Immunohistochemistry. Necdin-null mice (Gerard et al., 1999) were generated by heterozygous crosses, pregnant females were euthanized, and embryos were harvested at embryonic days 13.5 (e13.5) and 17.5 (e17.5). Genotypes were confirmed as previously described (Ren et al., 2003). Magel2-null mice were generated and genotyped as previously described (Bischof et al., 2007; Kozlov et al., 2007). Whole embryos (e13.5) or embryo heads (e17.5) were fixed in 10% acetic acid, 30% formaldehyde, 60% ethanol, overnight at 4°C and dehydrated in ethanol/water washes prior to embedding in paraffin. Paraffin embedding was performed by the UCSD Cancer Center Histology and Immunohistochemistry Shared Resource (http://cancer.ucsd.edu/Research/Shared/histology/index.asp). Sections were cut 10 μm thick on a microtome, floated onto SuperFrost Plus slides (Fisher) and dried overnight at 37°C. Approximately 120 to 250 sections were processed and stained for GnRH per head depending on the developmental stage. Prior to staining, slides were incubated at 60°C for 30 minutes Slides were deparaffinized in xylene washes, then rehydrated in ethanol/water washes. Antigen retrieval was performed by boiling for 10 minutes in 10 mM sodium citrate. After cooling and washing twice in water, endogenous peroxidase was quenched by incubating for 10 minutes in 0.3% hydrogen peroxide. Slides were blocked in PBS with 5% goat serum and 0.3% Triton-X-100 for 45 minutes. Slides were then incubated in anti-GnRH antibody (Affinity BioReagents PA1-121) diluted 1:1000 in blocking buffer overnight at 4°C and

incubated in biotinylated goat-anti-rabbit IgG (Vector Labs) at 1:300 for 30 minutes. GnRH peptide was visualized using the Vectastain ABC elite kit and VIP peroxidase kit (Vector Labs). Sections were counterstained using Vector Methyl Green (Vector Labs). Sections were analyzed using a Nikon Eclipse E800 microscope and images were taken with an Olympus camera. Counting of GnRH neurons was performed by examining all of the processed sagittal sections described above. Three or more embryos were analyzed per time point and genotype. Cells were divided into nasal, cribriform plate, and brain regions as shown in Figure 4-1A and the mean calculated. Error bars depict standard deviation.

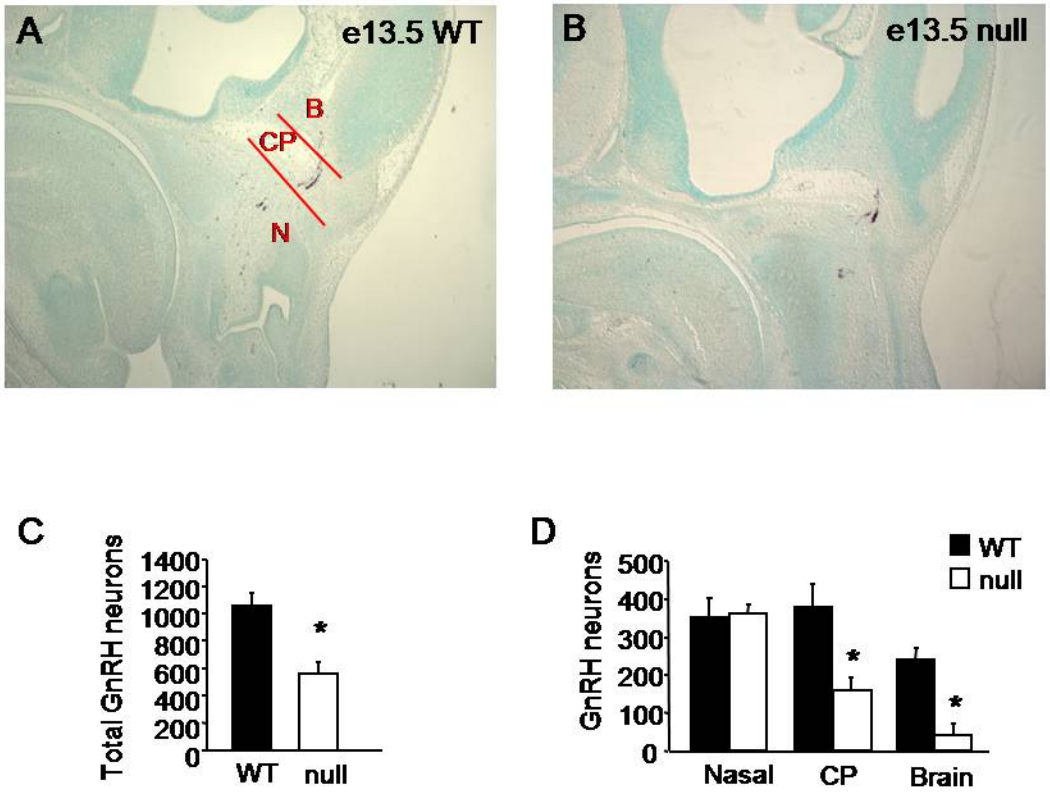

Figure 4-1. Embryonic day 13 necdin-null mice have fewer GnRH neurons. GnRH immunohistochemistry on e13.5 wild-type embryos (A) and Necdin null embryos (B). (C) Average e13.5 GnRH neuron numbers. (D) e13.5 GnRH neuron numbers in nasal [N], cribriform plate [CP], and brain [B] regions as depicted in (A). Immunohistochemistry photographs are shown at 4x. Means were compared by Student's T-test, compared to wild-type littermates and * indicates $p<0.05$. Error bars are standard deviation.

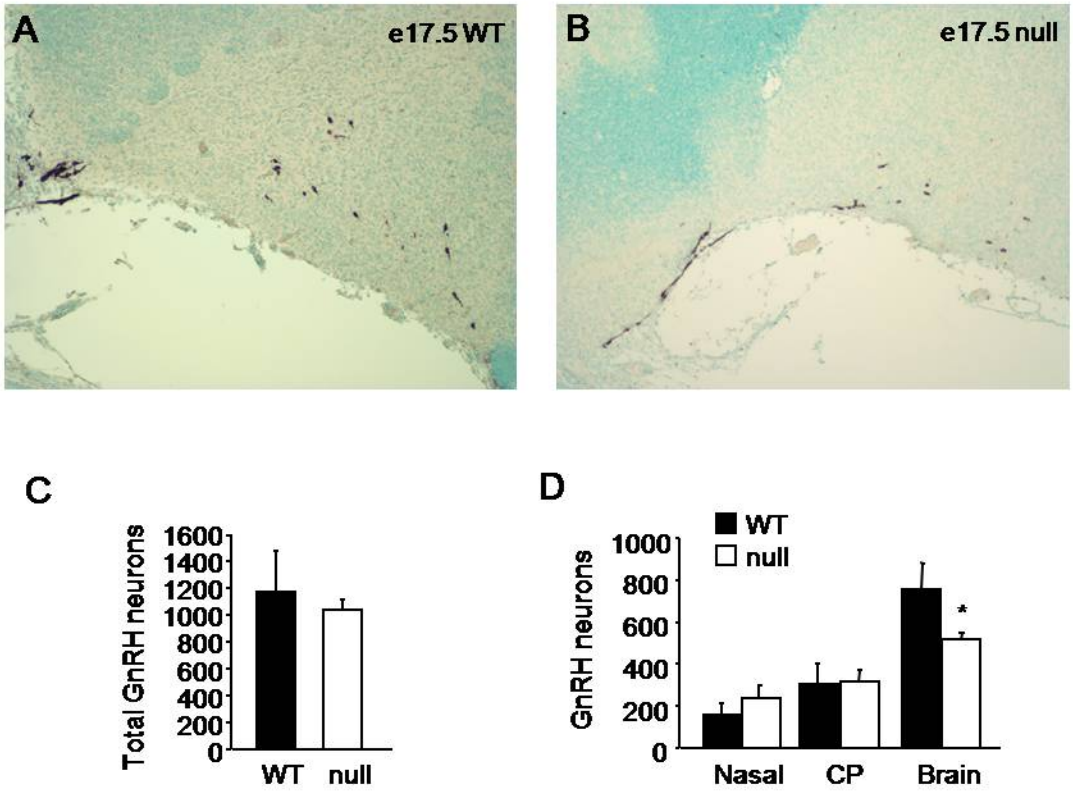

Figure 4-2. Embryonic day 17 necdin-null mice have reduced GnRH neuron numbers in the brain. GnRH immunohistochemistry on e17.5 wild-type embryos (A) and necdin null embryos (B). (C) Average e17.5 GnRH neuron numbers. (D) e17.5 GnRH neuron numbers in nasal, cribriform plate [CP], and brain regions as depicted in Figure 4-1A. Immunohistochemistry photographs are shown at 10x. Means were compared by Student's T-test, compared to wild-type littermates and * indicates $p<0.05$. Error bars are standard deviation.

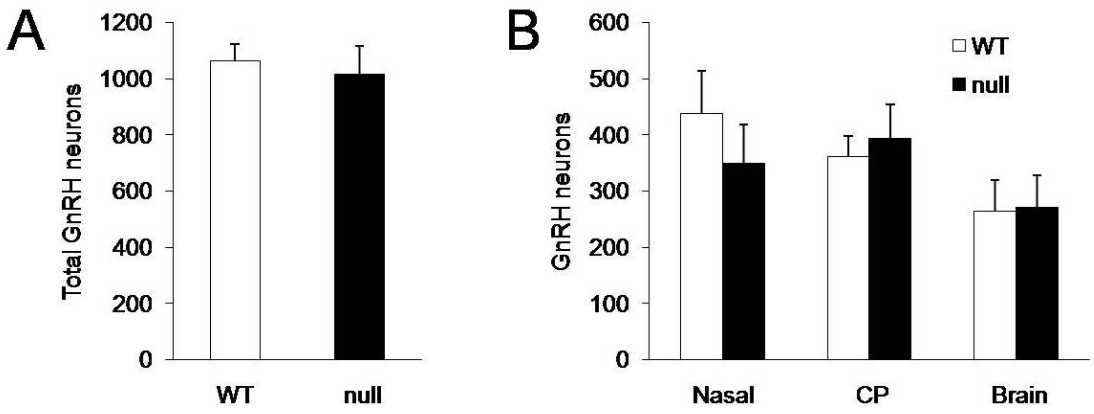

Figure 4-3. Magel2 null mice have normal numbers of GnRH neuron. GnRH immunohistochemistry was performed on e13.5 Magel2-null mice. A) Total numbers of GnRH neurons. B) GnRH neuron numbers in nasal, cribriform plate [CP], and brain regions as depicted in Figure 4-1A. These values represent the means of at least three animals from each genotype.

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The preceding chapter is, in part, a manuscript submitted for publication titled "Necdin, a Prader-Willi syndrome candidate gene, regulates development of gonadotropin-releasing hormone neurons" by Nichol L. G. Miller, Rachel Wevrick, and Pamela L. Mellon. The dissertation author is the primary researcher and author of this manuscript. Rachel Wevrick provided the mouse embryos for processing and analysis and also provided critical reading.

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CHAPTER V

CONCLUSIONS

The GnRH neurons are fundamental to the HPG axis, integrating input from the organism and its environment to send neuroendocrine signals to gonadotropes within the anterior pituitary, which execute their role in the axis to regulate the gonads, thus controlling reproductive function. For all of the events downstream of the GnRH neuronal signals to function properly, the GnRH neurons must develop in a unique and precise manner in which the spatiotemporal regulation of the GnRH gene, as well as developmental migration, is absolutely essential. Both *in vitro* (Mellon et al., 1990; Radovick et al., 1991) and *in vivo* (Lawson et al., 2002; Pape et al., 1999; Skynner et al., 1999b; Suter et al., 2000; Wolfe et al., 1996) models have been created to study the control and function of the GnRH neurons. While many studies have contributed to our understanding of the origin and development of the GnRH neuronal population (Livne et al., 1993; Schwanzel-Fukuda et al., 1992; Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a; Wray et al., 1994), the mechanisms by which GnRH is accurately expressed and the precise migration of the neurons remain unknown. Therefore, the aim of this dissertation is to expand our knowledge of factors that contribute to the restricted expression of GnRH and the unique developmental progression of the GnRH neurons.

At the initiation of my graduate studies, the regulatory regions that had been identified to promote neuron-restricted expression of GnRH included a 292 bp enhancer (located between -1863 and -1571, relative to the transcriptional start site)(Whyte et al., 1995) and a 173 bp proximal promoter ((Eraly and Mellon, 1995). These were confirmed *in vivo* with transgenic mice by using these elements to direct a β-galactosidase reporter (Lawson et al., 2002). However, while no ectopic expression of the reporter was observed, these elements alone did not target the entire population of GnRH neurons. This suggested that additional regulatory elements might be necessary to target the full and exact GnRH neuronal population. This led us to utilize the recent release of the sequences of the mouse and human genomes, in addition to the partially available rat sequence, to search for potential novel regulatory elements. By comparing upstream sequence across mammalian species, we identified a novel upstream enhancer (located between -2980 and -2631 in the rat sequence) (Givens et al., 2004). Concurrent with these studies, research using the mouse GnRH gene was published (Pape et al., 1999), demonstrating that increasing the regulatory sequence driving a transgene from -2.1 kb to -5.2 kb significantly increased the number of GnRH neurons that were targeted (from 60% to 89%), suggesting that sequence upstream of the enhancer and promoter could be important for *in vivo* regulation of GnRH. While we had identified the upstream enhancer as significant for neuron-restricted expression *in vitro* (Givens et al., 2004), we wanted to examine more distal sequence which corresponded to the mouse -5.2 kb. With the release of the entire rat

genome sequence, we compared up to -5 kb of the rat sequence with mouse and human, resulting in the identification of four novel regulatory elements (regions 1-4), one of which (region 1) lies just 5' to, and is likely to be an extension of, the characterized upstream enhancer. Two of these contribute to neuron-specific expression of the GnRH gene. Region 1 (in concert with the upstream enhancer) and region 3 are evolutionarily conserved enhancers and are likely to play important roles *in vivo* as well. Generation of a transgenic mouse model using these novel regulatory elements, in addition to the characterized enhancer and proximal promoter, would contribute insight into the role of these elements in GnRH gene regulation *in vivo*.

An additional means of GnRH regulation exists at the level of transcription factor binding of the regulatory elements. Many transcription factors have been shown to bind and regulate the GnRH gene (Belsham and Mellon, 2000; Clark and Mellon, 1995; Givens et al., 2005; Kelley et al., 2000; Lawson et al., 1996; Lee et al., 2001; Rave-Harel et al., 2004) and are illustrated in Figure 1-1. However none of these are restricted to GnRH neurons and so the means by which they specifically drive GnRH expression is not fully understood. We have identified NF1 as a transcription factor that functions cooperatively with Oct-1, a known GnRH regulator (Givens et al., 2004). Oct-1 DNA binding is also important for glucocorticoid hormone regulation of the GnRH gene (Chandran et al., 1999). It is possible that the stabilization action of NF1 on Oct-1 (Givens et al., 2004) is not only important for basal GnRH gene expression, but also hormonal response. Additionally, NF1 and Oct-1 can bind to several sites within the GnRH regulatory sequence, perhaps

allowing for some flexibility in their function. It will be interesting to determine whether the role of NF1 in GnRH regulation is as a co-factor only or whether it can also directly affect GnRH gene expression.

Co-factor binding to transcriptional regulators of GnRH is likely also contributes to dynamic regulation of GnRH promoter activity. We identified Grg proteins as co-repressors of Msx1 and Oct-1 (Rave-Harel et al., 2005). Grg proteins do not bind DNA directly, but instead function by regulating Msx repression or Oct-1 activation of GnRH. Additionally, a specific Grg protein, Grg5, can act as a dominant negative by binding other Grg proteins to inhibit their function. Thus, Grg proteins are able to contribute either positive or negative regulation to the GnRH gene. One can imagine that these types of co-factors could play a role in targeting GnRH expression by forming multi-protein complexes specific to GnRH neurons. The precise molecular mechanisms of these multi-protein complexes at the level of the DNA will be important to understand, and using techniques such as chromatin immunoprecipitation, they may be clarified in the future. The existence of so many means of transcriptional regulation of GnRH make it clear that restriction of GnRH gene expression is quite complex and requires many levels of control for the GnRH neurons to function properly.

Since GnRH is the central regulator of the HPG axis, and thus, reproduction, it is important to discuss its role in pathologies demonstrating reproductive abnormalities. While the hypogonadotrophic hypogonadism in Kallmann syndrome and many cases of idiopathic hypogonadotrophic hypogonadism have been attributed

to a deficiency in GnRH, the cellular and molecular basis for the lack of GnRH is not entirely understood. However, mutations in genes such as FGFR1 and KAL1 have provided clues, and demonstrated that proper GnRH neuronal development is essential for reproductive function. Less clear are contiguous gene disorders, such as Prader-Willi syndrome, where a myriad of symptoms ranging from infantile hypotonia to subsequent hyperphagia, developmental delays, and hypogonadism are present within patients containing multigenic mutations leading to loss of expression of several genes. One theory is that loss individual genes gives rise to specific phenotypes observed, which contributes to the wide array of characteristics. We have demonstrated here that lack of necdin (a Prader-Willi candidate gene) expression, results in improper GnRH gene expression and neuronal development. Necdin over-expression activates GnRH gene transcription and knock-down of necdin results in decreased levels of GnRH, demonstrating both its sufficiency and necessity, *in vitro*. Mouse embryos lacking necdin expression were examined and found to have reduced numbers of GnRH neurons, indicating the importance of necdin for GnRH neurons, *in vivo*, as well. Interestingly, previous research observed that necdin can exist *in vitro* within protein complexes with Msx and Dlx, which are characterized homeodomain protein regulators of GnRH gene expression (Givens et al., 2005). Msx repressors and Dlx activators both bind to the same sequences in the GnRH regulatory regions and are expressed along the GnRH migratory path. The two GnRH neuronal cell lines, GT1-7, representing a mature GnRH neuron with high GnRH levels, and Gn11, representing an immature GnRH with low levels of GnRH expression, both express

Msx family members, so it is unclear how Msx binding to the DNA is reversed so that Dlx can bind and activate the GnRH gene as development progresses. Msx homeodomain proteins are potent transcriptional repressors that play many roles in biological processes (Bendall and Abate-Shen, 2000) and, therefore, it is important that this repression is relieved when necessary during development for activation of the GnRH gene. We show that necdin relieves Msx1 repression of the GnRH gene, and that this is occurring through the characterized Msx/Dlx binding sites. Interestingly, necdin did not affect Dlx activation of GnRH. We also observed endogenous protein complexes containing both necdin and Msx1, indicating a functional interaction may exist within GnRH neurons. These studies have led us to hypothesize that the molecular mechanism responsible for removing Msx repressor homeodomain transcription factors from the GnRH regulatory elements may involve necdin. As necdin has been shown to play a role in neuronal development in other systems (Andrieu et al., 2003; Kuwako et al., 2005), it is reasonable to propose that necdin could be doing so in the development of the GnRH neurons, however the mechanism of relieving Msx repression has not been reported. Interestingly, the Msx homeodomain transcription factor has been shown to regulate the development of several tissues including tooth, bone, limb, anterior forebrain, and other craniofacial structures (Lallemand et al., 2005; Satokata et al., 2000; Satokata and Maas, 1994). It would be interesting to assess whether necdin is expressed and active in these tissues and, if so, whether it plays a role regulating Msx repression.

During the last several years in which this research was taking place, many studies have shed light on the regulation and development of the GnRH neuron. The material presented herein provides evidence regarding the control of the GnRH neuron, using both *in vitro* and *in vivo* models, which will complement the recent published reports. Chapter II addresses the identification of novel evolutionarily-conserved regions, how they contribute to neuronal cell-specificity of GnRH expression and transcriptional regulators that bind them to regulate GnRH gene activity. Chapter III identifies a novel regulator of GnRH, the MAGE family member, necdin, that acts antagonistically to the Msx homeodomain transcription factor. Chapter IV focuses on the role of necdin in the developmental migration of the GnRH and, along with Chapter II, provides an potential explanation for the source of reproductive abnormalities in Prader-Willi syndrome.

In the larger view, it is interesting to set these findings within the framework of other known causes of hypogonadotropic hypogonadism resulting from defects in GnRH neurons. Several genetic sources have been identified, including mutations in the *KAL1* and *FGFR1* genes, leading to Kallmann's syndrome. However, many cases are idiopathic in nature, and their genetic basis remains a mystery. Conversely, Prader-Willi syndrome is a multigenic neurodevelopmental disorder with a wide array of diagnostic characteristics, one of which is hypogonadotrophic hypogonadism. As a large area of the chromosome is affected and such varied symptoms are observed, it has been difficult to decipher the precise etiology of the disease. However, our findings indicate that loss of necdin expression in Prader-Willi patients could be

responsible for their hypogonadotrophic hypogonadism, explaining a portion of the disorder. Additionally, this leads to contemplation of whether other cases of idiopathic hypogonadotrophic hypogonadism could be the result of a lack of functional necdin or whether individuals with undiagnosed reproductive defects possibly carry mutations in the necdin gene. The better our understanding of the genetic and physiological basis of various reproductive disorders, the better the treatment options that will be available to patients.

In summary, the research presented herein contributes to our understanding of the mechanisms involved in the regulation of the GnRH gene and how this affects the GnRH neuron, *in vivo*. It is our hope that this research will improve our knowledge of the molecular and cellular control of reproduction.

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