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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Ventral Anterior Homeodomain Protein Regulates
Gonadotropin-Releasing Hormone and Fertility

A Thesis submitted in partial satisfaction of
the requirements for the degree Master of Science

in

Biology

by

Anika Tamrazian

Committee in charge:

Professor Pamela L. Mellon, Chair
Professor Randolph Y. Hampton, Co-Chair
Professor Deborah L. Yelon

2014

The Thesis of Anika Tamrazian is approved and it is
acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2014

Dedication

I dedicate this thesis to my dear friends and family
for their continued support and encouragement throughout the years.

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Abbreviations

D	Diestrus
E	Estrus
FSH	follicle stimulating hormone
E1	GnRH enhancer 1
E2	GnRH enhancer 2
E3	GnRH enhancer 3
GnRH	gonadotrophin-releasing hormone
HET	Heterozygote
HPG	hypothalamic-pituitary-gonadal
IHH	idiopathic hypogonadotropic hypogonadism
KO	Knock out
LH	luteinizing hormone
M	Metestrus
P	progesterone
P	Proestrus (in chapter 1) or GnRH promoter (chapter 2)
T	testosterone
Vax1	Ventral anterior homeobox 1
WT	Wild type

ABSTRACT OF THE THESIS

Ventral Anterior Homeodomain Protein Regulates
Gonadotropin-Releasing Hormone and Fertility

by

Anika Tamrazian

Master of Science in Biology

University of California, San Diego, 2014

Professor Pamela L. Mellon, Chair

Professor Randolph Y. Hampton, Co-Chair

Idiopathic Hypogonadal Hypogonadism (IHH), a condition associated with the loss of gonadotropin releasing hormone (GnRH) neurons and/or disruption of the hypothalamic pituitary gonadal axis (HPG), leads to complete infertility and abnormal reproduction. Currently, a large number of the mutations involved with IHH are of unknown origin, but are likely to arise from polygenic mutations. We here describe Ventral Anterior Homeobox 1 (Vax1) as a potential genetic contributor to IHH. Vax1 heterozygote mice show numerous characteristics related to subfertility including smaller litters, delayed estrous cycling in females, and reduced sperm quality in males. The importance of GnRH neurons for reproduction and development led us to investigate the regulatory role of Vax1 in GnRH transcription, which determined Vax1's ability to

repress GnRH transcription through the -41bp binding site of the GnRH promoter. cFos, an immediate early gene, has been known to repress GnRH transcription through AP-1 sites on the GnRH promoter. The possible interaction between Vax1 and cFos was also considered to further determine the molecular mechanism of Vax1 regulated repression of the GnRH promoter.

Introduction

Infertility

Approximately 11% of women and 7.5% of men of reproductive age are infertile (<http://www.cdc.gov/reproductivehealth/Infertility/Index.htm>). Infertility can be caused by a variety of factors including genetic, environmental, and hypothalamic/pituitary defects. These factors may cause lack of sex hormones that can be attributed to deficiencies of regulatory reproductive hormones along the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis has a regulatory feedback mechanism that utilizes many reproductive hormones such as hypothalamic gonadotropin-releasing hormone (GnRH), pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH), as well as gonadal sex hormones including estrogen, progesterone and testosterone (Illustration 1). GnRH neurons are scattered along the hypothalamus, and release GnRH in a pulsatile manner. GnRH pulses act upon the target pituitary gland to release LH and FSH in a specific pattern. LH and FSH signal the gonads to release sex hormones (testosterone, estrogen and progesterone) and are capable of initiating feedback mechanisms throughout the axis (Illustration 1).

A condition known as Idiopathic Hypogonadotropic Hypogonadism (IHH) is associated with the disruption of GnRH neuron migration and development and leads to complete infertility. Sixty percent of IHH patients are also anosmic, and show characteristic loss of olfaction, otherwise known as Kallmann syndrome (1). Gene mutations associated with Kallmann syndrome encode proteins that regulate GnRH neuronal migration (Illustration 2). These mutations are capable of disrupting proper migration of GnRH neurons to the hypothalamus, causing abnormal GnRH neuron counts leading to infertility (Illustration 1, Illustration 2).

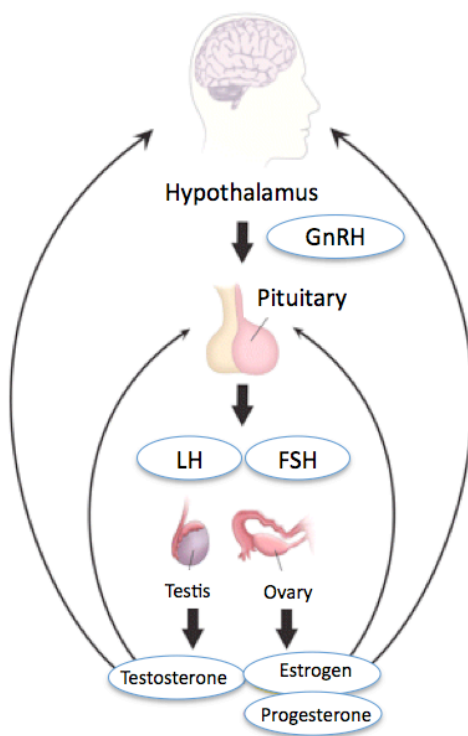


Illustration 1. The hypothalamic-pituitary-gonadal axis

GnRH neurons in the hypothalamus regulate downstream reproductive hormones including LH and FSH released from the pituitary gland. These hormones signal the gonads to release sex hormones (progesterone, estrogen and testosterone), a requirement for proper reproductive function.

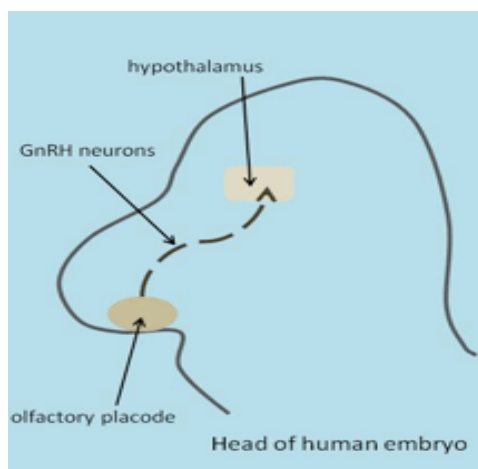


Illustration 2. GnRH neuron migration in development

GnRH neurons arise in the olfactory placode. During development, these neurons migrate from the forebrain to the hypothalamus and become mature GnRH neurons (1, 2).

Studies have shown that most of the genetic mutations causing IHH are unidentified (1). Genetic mutations known to cause IHH are either autosomal recessive or dominant. Based on this, it was recently proposed that a number of the IHH cases with unknown origin might be the results of compound heterozygosity. Although compound mutations are harder to detect, as they require the identification of more than one gene mutation, numerous descriptions of haploinsufficiency adversely affecting fertility have been reported in rodents and humans (3-6).

Transcriptional Regulation of the GnRH Promoter

GnRH release is required for fertility (Illustration 1); therefore, investigation of the regulation of the GnRH promoter is essential for understanding infertility. The GnRH promoter

consists of three enhancer regions (E1, E2 and E3) and a promoter (P) that specify GnRH gene expression to differentiated and mature GnRH neurons (Illustration 5) (7). Interestingly, E1 along with the proximal GnRH promoter are sufficient to specify GnRH transcription to GnRH neurons in transgenic mice (8), making these two regions of particular interest.

Many transcription factors have been identified as directly regulating the GnRH promoter, these include both immediate early genes as well as homeodomain binding transcription factors. Homeodomain transcription factors regulate gene transcription by bind to ATTA sites in target genes promoters. In adult tissue, this family of transcription factors tend to be expressed continuously in very specific cells at constant levels over time, *their expression can be considered relatively stable*. On the other hand, immediate early genes (IEG) are characterized by having low basal levels, and appropriate stimuli will rapidly and efficiently increase their transcription and translation. *IEG are thus a system the cells have to respond rapidly to stimuli*.



Illustration 3. Enhancer regions of the GnRH promoter

Previously, in the Mellon laboratory, we have found Ventral Anterior Homeobox (Vax1) gene expression in GnRH neurons (Illustration 5). Vax1 is a homeodomain transcription factor regulating transcription of its target genes through binding to ATTA sites (9). As a model for mature GnRH-secreting neurons, we use the GT1-7 cell line (10). It is very difficult to study

GnRH neurons *in vivo* as they are scattered through the hypothalamic area (2). This makes the GT1-7 model a great tool to study transcriptional regulation of GnRH neurons, as they can be easily cultured and transfected *in vitro*. The GT1-7 cell line was created by targeting tumorigenesis to specific hypothalamic neurons in the mouse, thus generating neuronal tumors that were purified, cultured, cloned, and characterized (10).

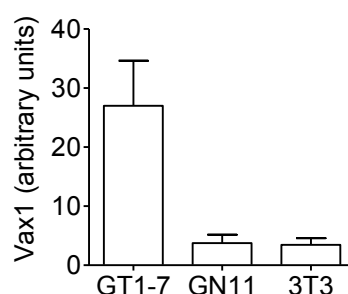


Illustration 4. Vax1 is expressed in GnRH neurons

Mouse Vax1 transcript levels as determined by qRT-PCR of total RNA obtained from mature GnRH neurons (GT1-7), immature GnRH neurons (GN11) and fibroblast (3T3).

Initial studies in the Mellon laboratory have found that Vax1 represses endogenous GnRH transcription (Illustration 5), as well as GnRH enhancer 1 and promoter (GnRH E1/P) luciferase driven transcription (Illustration 3). Vax1 required both the GnRH E1 and P to repress GnRH E1/P driven luciferase transcription. To determine if Vax1 needed to bind directly to the GnRH promoter, ATTA sites in the GnRH E1/P-luciferase reporter vector were mutated. Studies showed that Vax1 was able to repress GnRH transcription on the wildtype (WT) reporter. However, when either the E1 or P ATTA sites were mutated, Vax1 lost the ability to repress GnRH. To prove that Vax1 can bind directly to the GnRH promoter we studied protein –DNA interaction by electrophoretic mobility shift assay (EMSA). It was then determined that Vax1

repressed GnRH transcription by binding directly to two ATTA sites located at -41 bp and -1640 bp of the promoter and enhancer regions, respectively.

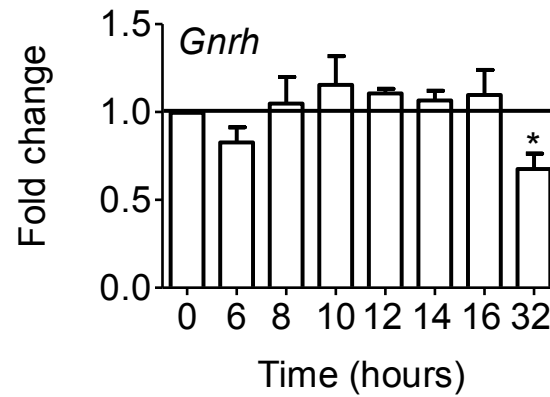


Illustration 5. Vax1 overexpression represses endogenous GnRH mRNA

Vax1 was overexpressed in GT1-7 cells and cells were harvested over a time course from 0-32 h after the end of transfection. GnRH transcript levels were determined by qRT-PCR.

Chapter 1

Effects of Vax1 on Fertility and Reproduction in Mice

I. Introduction

We were interested in identifying new genes that could be involved in composite IHH. Initial work in the laboratory had shown that Ventral anterior homeobox 1 (Vax1) was expressed in GnRH neurons (Illustration 5). Vax1 is a homeodomain transcription factor that affects ventral forebrain organization in rodents and humans (11). Vax1 is expressed in the ventral forebrain and hypothalamus, the region where GnRH neurons migrate to during development. In addition, Vax1 mice possess additional secondary phenotypical characteristics of IHH, *i.e.*, cleft lip (12), a gene being expressed in the ventral forebrain during GnRH neuron development and Vax1 KO mice have no GnRH neurons in the brain at embryonic day17.5, making it a potential candidate in IHH. Unfortunately, Vax1 KO mice cannot be studied as they die soon after birth (13).

Based on this evidence, we hypothesized that Vax1 mutations may cause disruptions in GnRH release, and possibly affect normal reproduction. To address this question, we characterized the phenotypic manifestations of Vax1 heterozygous (HET) male and female mice. We determined that both genders are subfertile and have fewer GnRH neurons. We propose that Vax1 is a candidate gene in composite IHH.

II. Materials and Methods

Mouse Colony

All animal procedures were performed in accordance with the UCSD Institutional Animal Care and Use Committee regulations. Mice are housed on a 12 hour light/12 hour dark cycle with *ad libitum* chow and water. Vax1 knock-out (KO) mice were provided by Dr. Bharti (National Institutes of Health, Bethesda, MD). Mice were sacrificed using CO₂ overdose followed by cervical dislocation.

Genotyping

Genotypes were determined using PCR pre mix (Bioneer). To determine genotype the following primers were used Vax1 WT /KO F (5'gaaacgtgggggctgcgaaat 3'), WT R (5'gaaacgtgggggctgcgaaat 3') and KO R (5'gctcccgattcgcagcgcacg-3') generating a Vax1 WT PCR fragment of ~550 bp and a Vax1 KO PCR fragment of ~750 bp.

Fertility study

Adult, virgin, wildtype (WT) or HET mice were paired in cages. The litter size and number of litters born were recorded over a 120-day period.

Plug efficiency

Adult (10-16 weeks of age), virgin, male and female mice were housed together and plug formation was monitored every day between 9-10 am over a period of 10 days. The number of days it took for the male to plug the female was recorded.

Pubertal onset

All mice were inspected daily for pubertal onset: vaginal opening for females and preputial separation for males. Pubertal onset inspection started when mice were 22 days of age.

Estrus cycling

Estrous cycles of female mice were determined using vaginal smears performed between 9-11 am daily on 3-5 month old mice. Smears were collected on glass slides and counterstained with 0.1% methylene blue (Spectrum, Gardena, CA). Cell type was determined using microscopy.

Sperm collection and count

Sperm was collected from epididymis of male mice in M2 media. Epididymis was cut in half and sperm were expelled by gently pressing down on the epididymis. The numbers of total and motile sperm were counted using a hemocytometer. To immobilize motile sperm, the hemocytometer was placed on a heat block for 5 min at 55°C. The second epididymis was cut into small pieces and left 30 min at room temperature in M2 media. The solution was filtered into a falcon tube and sperm were diluted with PBS before counting total numbers of sperm.

H&E staining of testis

The testes were collected from WT and HET mice using surgical techniques. They were then fixed using a formaldehyde solution and embedded in paraffin. 10 micrometer sections were cut using the microtome. Paraffin was melted using an oven at a high temperature setting, and stained using hematoxylin and eosin (H&E).

III. Results

Vax1 Female Mice Have Irregular Estrus Cycles

We characterized the reproductive phenotype of Vax1 heterozygous (HET) mice, assessing various aspects of their reproductive function.

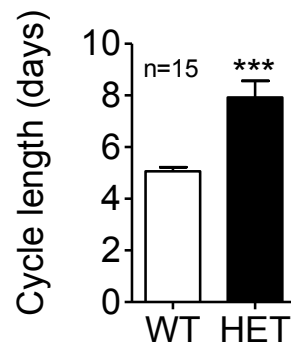


Figure 1. Vax1 female HET mice have prolonged estrus cycles

Average cycle length in WT and HET mice, n=15. Statistical analysis by Student's t-test as compared to control; *p<0.05; **p<0.01.

Instead of the human menstrual cycle, mice have estrous cycling. The mice go through four different stages that can be determined visually via differences in cell morphology. To determine if female mice had irregular estrous cycles, we monitored cycle length via vaginal lavage. Results indicated that Vax1 HET mice completed a full cycle in 7-9 days compared to the WT littermates that took about 5 days (Figure 1), indicating a cycle irregularity within Vax1 HET female mice. To determine which part of the estrous cycle was responsible for the increased cycle length, we compared the time spent in each stage of the cycle between Vax1 WT and HET mice.

We found that Vax1 HET mice spent significantly more time in metestrus and less time in diestrus than WT littermates (Figure 2, Figure 3).

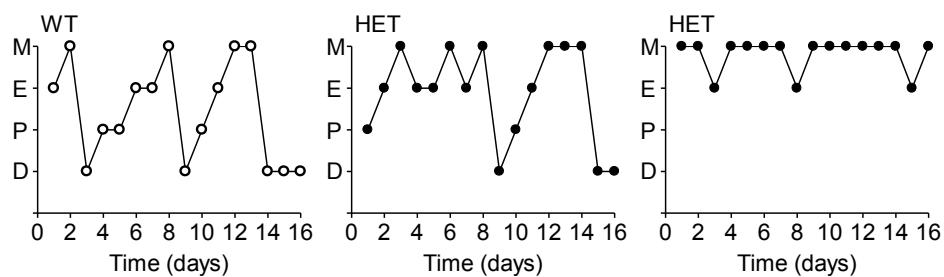


Figure 2. Estrous cycling of WT and HET Vax1 mice

Estrous cycling was monitored daily in Vax1 WT and HET mice. M: metestrus, E: estrus, P: proestrus, D: diestrus.

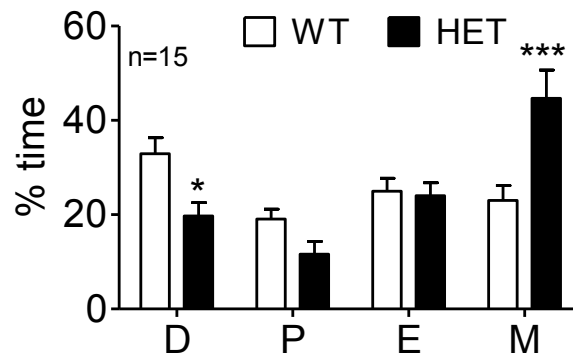


Figure 3. Percent time spent in each stage of the cycle

The percent time WT and HET mice spend in diestrus (D), proestrus (P), estrus (E), metestrus (M), n=15. Statistical Analysis done by Two-Way ANOVA followed by Bonferroni; *p<0.05;

p<0.01; *p<0.001 as compared to WT in the same stage of the cycle.

Vax1 Heterozygous females take longer to mate

Males have the capacity to “plug” a female, indicating successful copulation. This plug is easily detectable and can be monitored. Male mounting was evaluated through vaginal plug formation in the female to determine subfertility characteristics in Vax1 mice. Results indicated that WT males took on average 3 days to plug a WT female, which was also seen in HET males paired with WT females. However, both Vax1 HET and WT males took on average 5-7 days to plug a HET female (Figure 4). Female mice are only receptive to males during the late proestrus/early estrus stage of the estrous cycle, indicating that female mice provided a greater contribution to plugging inefficiency than the male mice.

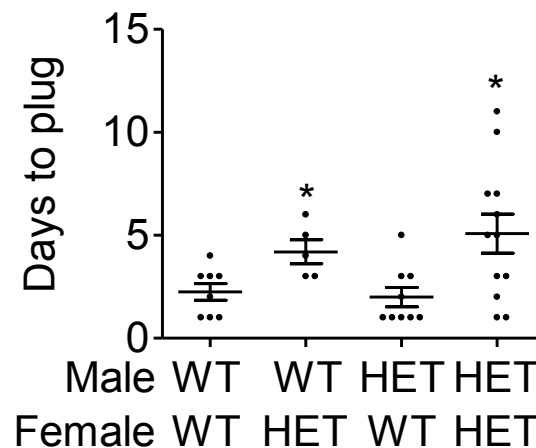


Figure 4. Days to first plug in Vax1 WT and HET mice

Fertility assessment of Vax1 HET mice. Virgin male and female mice housed together and number of days to first plug was monitored. Data represent means $\pm$ SEM. Statistical analysis by Students t-test as compared to WT x WT, * $p < 0.05$; ** $p < 0.01$.

Vax1 Male and Females HET mice are subfertile

To evaluate the fertility of Vax1 HET mice, the number of days to the first litter was recorded. The time to first litter of female Vax1 HET mice was slightly, but not significantly (Student's t test, $p = 0.0826$) increased as compared to the WT x WT matings (Figure 5). The number of litters produced by the HT female matings in the first three months of mating and litter size were both significantly reduced compared to the WT x WT matings (Figure 5, Figure 6). WT mice produced approximately 2.5 litters in 3 months (Figure 5), whereas the HET female mice produced about 1.5, again correlating with decreased fertility stemming from irregular and prolonged estrous cycling. Additionally, HT males produced normal litter numbers in 3 months (Figure 5), but the size of the litters was reduced (Figure 6) suggesting a problem with the sperm.

To determine if the origin of Vax1 HET male subfertility was associated with sperm generation, we evaluated sperm morphology. We found that sperm morphology in Vax1 HET mice was comparable to WT (Figure 8); however, sperm quality assessments determined that there were fewer total and motile sperm than in the WT (Figure 8).



Figure 5. Number of days to first litter in Vax1 WT versus HET mice

The number of days to produce the first litter was monitored in Vax1 WT and HET mice. The cut off was 120 days, n=5-12. Data represent means $\pm$ SEM. Statistical analysis by Student's t-test as compared to WT x WT, * $p < 0.05$; ** $p < 0.01$.

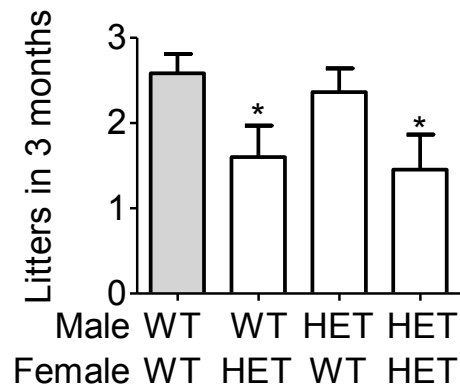


Figure 6. Number of litters in three months in Vax1 matings

The number of litters produced by Vax1 HET matings was recorded (n=10-13). Data represent means $\pm$ SEM. Statistical analysis by Student's t-test as compared to WT x WT, * $p < 0.05$.

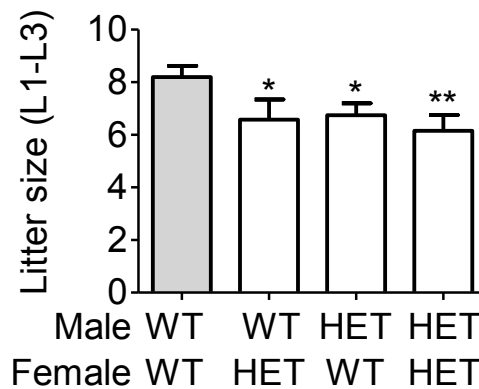


Figure 7. Average litter size of first three litters in Vax1 matings

The average litter size of the first three matings was recorded for Vax1 WT and HET matings. Data represent means $\pm$ SEM. Statistical analysis by Student's t-test as compared to WT x WT, * $p < 0.05$; ** $p < 0.01$.

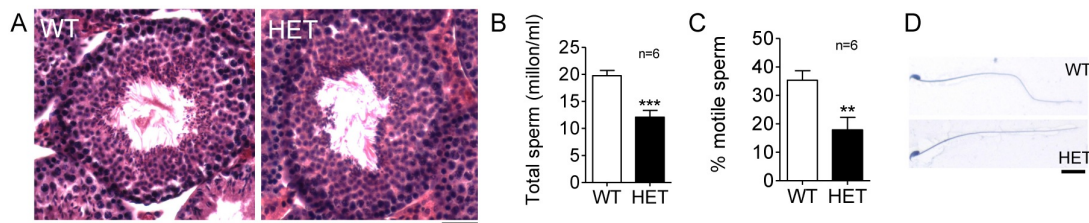


Figure 8. Male Vax1 mice showing reducing sperm count and motility

A) H&E stained testes. Scale bar indicates 10 μ m. B) Total sperm count per epididymis and C) % of motile sperm, n=6. D) H&E stained sperm. Scale bar indicates 1 μ m. Data represent means $\pm$ SEM. Student's t-test as compared to control; **p<0.01; ***p<0.001.

IV. Discussion

Studies have shown that the reproductive phenotype of Idiopathic Hypogonadotropic Hypogonadism is linked to mutations in a number of genes (1). Some of these mutations have been attributed to lack of GnRH migration, usually associating with Kallmann Syndrome, and HPG axis defects. In IHH patients, the disruption of GnRH neuron migration to the hypothalamus, along with lack of regulation of the HPG axis, leads to infertility. Many genes involved in ventral forebrain development, the site of GnRH migration, are contributors to cleft palate formation, an abnormality seen in Kallmann patients. Vax1 mutations have been linked to lack of GnRH migration during development, and this correlation has been supported in current studies showing at least 50% loss of GnRH neurons in the hypothalamus in Vax1 adult HET mice (data not shown). We have seen subfertility characteristics in both male and female Vax1 HET mice that are most likely due to fewer GnRH neurons present in the hypothalamus.

The corpora lutea is a temporary structure within the ovary that remains after ovulation occurs. Vax1 female ovaries presented fewer corpora lutea structures than those of the WT

animal (data not shown). With this information, we were compelled to study the estrous cycle of Vax1 HET mice. Results showed that Vax1 female HET mice spent on average more time in metestrus, and had longer cycle length, as they were unable to advance through the stages of the cycle in a timely manner. Additionally, we observed that when Vax1 HET females were paired with either Vax1 WT or HET males, plugging efficiency decreased and the mice took longer to plug. We then showed that there is a problem with the female estrous cycling, as the females remain in metestrus for a longer time frame. Since female mice are only receptive during the estrus stage of the cycle, we can understand why Vax1 HET females lack efficiency with male plugging.

Fertility assessments concluded that Vax1 HET mice took longer to produce their first litter, had fewer litters in three months, and had a smaller litter size. Similarly with plugging efficiency studies, when Vax1 HET females were paired with Vax1 WT or HET males, the mice took longer to produce the first litter. This can be due to irregular estrous cycling that leads to inefficient plugging as seen in Figure 4. Also, taking longer to produce the first litter would lead to fewer litters being produced in the span of three months, as seen in Figure 6. Interestingly, we noticed that all Vax1 matings in the litter size study consistently showed reduced number of pups. Again, in the female, this correlates with the reduced number of corpora lutea. However, when the HET males were placed with the WT or HET females, litter size was significantly reduced as well. These data suggest a possible defect in sperm morphology, counts or motility. We can see in Figure 8, a significantly reduced number of total sperm was found in Vax1 HET mice as compared to the WT. Also, the percent motile sperm was significantly decreased in Vax1 HET mice. All other morphology was normal. Thus, the smaller litter size could be attributed to the reduced sperm counts.

The subfertility characteristics we have seen in Vax1 HET mice indicate that this gene might serve as a possible candidate in IHH. IHH, a disorder of fertility, has been characterized by several mutations of genes in an additive effect that leads to complete lack of fertility.

Chapter 2

cFos and Vax1 Regulation of the GnRH promoter

I. Introduction

Since GnRH is an important component of the HPG axis regulating reproduction (Illustration 1), we have studied the GnRH promoter controlling GnRH transcription. Previous studies have shown that cFos, an immediate early gene induced by neuronal activation (14), is a repressor of GnRH transcription through binding to the -94 and -1727 bp of the GnRH promoter (15, 16). Interestingly, Vax1 overexpression in GT1-7 cells also resulted in increased cFos transcript levels prior to repression of GnRH (Illustration 6, compare to Illustration 5).

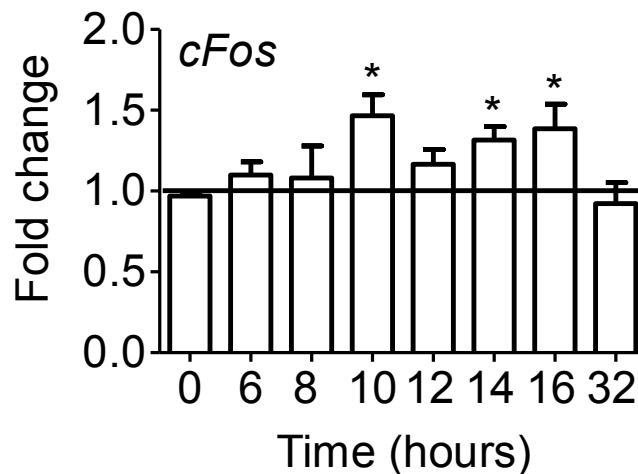


Illustration 6. cFos levels in Vax1 overexpressed cells.

Vax1 was overexpressed in GT1-7 cells and cells were harvested from 0-32h after the end of transfection. cFos transcript levels were determined by qRT-PCR. Statistical analysis by Student's t-test, each time point is compared to its own empty vector control; * $p < 0.05$.

Due to the time frame of the overexpression study (Illustration 5), and the fact that cFos is an immediate early gene, thus being transcribed and translated to protein rapidly in response to neuronal activation, it is possible that the repression of GnRH transcription observed after Vax1 overexpression is a combination of 1) direct binding of Vax1 to the GnRH promoter repressing its transcription, and 2) Vax1 induced cFos expression followed by cFos repression of the GnRH promoter (Illustration 7).

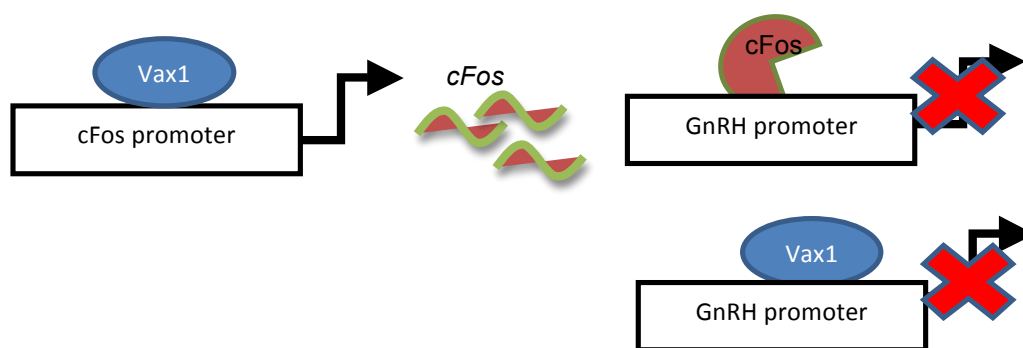


Illustration 7. Hypothetical Model 1 of Vax1 and cFos regulation of the GnRH promoter

This hypothetical model depicts our understanding of Vax1 and cFos regulation of the GnRH promoter. It is understood that Vax1 binds to the GnRH promoter, repressing transcription. However, Vax1, also increases cFos transcription. cFos at its turn acts upon the AP-1 site on the GnRH promoter to further repress GnRH transcription.

Since cFos, like Vax1 is a repressor of GnRH transcription (14-16) (Illustration 5), we wanted to study a potential cFos/Vax1 interaction, and its effect on GnRH transcription. To do this, GT1-7 cells were transfected with mutations of either the enhancer or promoter region of GnRH E1/P-luciferase, and then we determined the capacity of Vax1 and cFos to regulate its transcription.

Previous studies have shown that within the timeframe where Vax1 represses GnRH transcription (Illustration 5), an immediate early gene known as cFos is activated (Illustration 6). cFos, once activated, interacts with cJun to form a heterodimer called AP1 that works as the transcriptional complex. cFos and Vax1 exhibit nearby binding sites (Illustration 8), thus we have hypothesized that Vax1 and cFos interact with each other to regulate GnRH transcription.

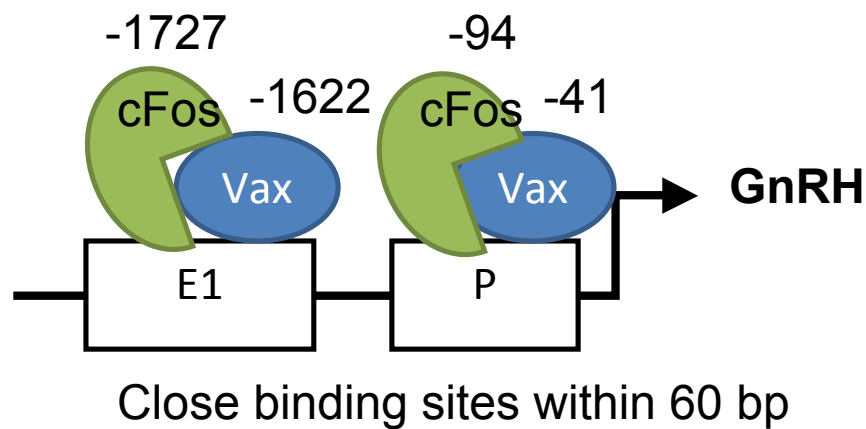


Illustration 8. Hypothetical model 2: Vax1 and cFos regulation of the GnRH promoter

involves interaction between these two transcription factors

In this study we describe a complex interaction of cFos and Vax1 on the GnRH promoter suggesting a possible physical interaction between these two transcription factors.

II. Materials and Methods

Cell Culture

GT1-7 or Cos cells were cultured in cell culture dishes (Nunc, Denmark) using Dulbecco's modified Eagle's medium (DMEM) with the addition of 10% Foundation B fetal bovine serum (FBS) and 1x penicillin streptomycin. Cells were split using trypsin in PBS and media changed approximately every 3-5 days.

Electrophoretic Mobility Shift Assay (EMSA)

Transfection and collection of cells: Cos cells (50,000 cells/ml) plated on 10 cm petri dishes (Nunc) were transfected with a final concentration of 200 pg/ μ l of Vax-1 Flag plasmid (Origene) or its empty vector, pCMV6 (Origene) using Fugene following manufactures instructions. After a 48 hour incubation time, the media was vacuumed off and washed with 1x cold PBS. The cells were lysed using 750 μ l of cell lysis buffer (1M Tris pH 7.4, 500mM NaCl, 1M MgCl₂, 100mM PMSF (in ETOH), 1mg/ml sigma protease inhibitors, 1M NaF, H₂O). Cell lysate was transferred to a 1.0 ml microcentrifuge tube and left to swell on ice for 15 minutes. The cells were gently passed through a 25 5/8 gauge needle four times and then centrifuged at 1700 g at 4 °C to pellet the nuclei. The supernatant was aspirated off and resuspended in hypertonic buffer (1M Hepes pH 7.9, 50% glycerol, 1M KCL, 1M MgCl₂, 100mM PMSF in ETOH, 1mg/ml sigma protease inhibitors, 1M NaF, 0.1M EDTA, 0.1M EGTA. The nuclei were left on ice for 30 minutes and centrifuged at 4 °C for 10 minutes at 20,000 g. The supernatant was aliquoted (10 μ liter/tube) and placed at -80 °C.

Annealing Oligo Probe: Oligos (IDT, San Diego, CA) were diluted in NaCl and H₂O to make 100 μ l of 10 μ M in 50 mM NaCl (forward +reverse strands). It was boiled at 100 °C for 5-10 min and cooled at room temperature for at least 1 hour. The probe was stored at -20 °C.

Labeling the probe: The probe was labeled using Gamma-ATP 32 making 100 fmol/ μ l radio-labeled double stranded oligo (H_2O , 10x T4 polynucleotide Kinase buffer, double stranded oligo, T4 polynucleotide kinase). The probe was incubated at 37 °C for 1 hour and then purified using Biorad Micro Bio-Spin 6 Columns. Reactions were set up using buffer (10X GSB, 5% BSA, 0.1 M DTT, 0.1M PMSF, 350 ng/ μ l poly dIdC), 2 or 3 μ g nuclear extract, rabbit anti-flag (Origene) antibody, rabbit IgG, unlabeled competitor and water. Eighteen μ l of sample was loaded into each well of a 5 % gel (5 % 29:1 acrylamide, 32.5 mM Tris base, 11.25 mM boric acid, 0.625 mM Na_2EDTA), and run for 2-3 hours at 250 V. The gel was following transferred and dried onto Watman paper for 2h at 80 °C under vacuum. The Watman paper with the dried gel was placed in a cassette and exposed on Kodak film for 1-5 days at room temperature.

Western Blot

Samples were obtained as described in *EMSA Transfection and collection of cells*. Protein concentration was determined via Bradford assay reagent (Bio-rad laboratories). 10 μ l of protein was loaded per well in a 10% SDS-PAGE (sodium dodecyl polyacrylamide gel electrophoresis). The gel was run in a separation buffer (1 M tris pH 8.8, 0.4 % SDS) for approximately 2h at 120V. Transfer buffer (0.05 M tris, 0.04 M glycine, 0.04 % SDS, 20% methanol) was used to transfer the gel onto a nitrocellulose membrane running at 400 mA for 1 hour at 4 °C. Membranes were washed 3 times for 10 minutes in 0.05% PBS-Tween (PBS-T) using the shaker at RT. Membrane was blocked using 3% milk in 0.05% PBS-T for 60 min at RT. Membranes were washed in PBS-T for 3x5 min. Primary antibodies (1:3000 rabbit anti-Flag, 1:1000 GAPDH-HRP, 1:500 rabbit anti TATA binding protein) were added and left O/N on shaker at 4 °C. Membranes were washed 3x10 min in PBS-T at RT. The membranes were incubated for 1 hour with anti-rabbit HRP antibody (1:1000 in 1% milk in PBS-T) at RT under

agitation. The bands were detected using a luminol chemoluminescent solution (1:1) and revealed in a FluorChem Q.

Luciferase Assay

Approximately 24 hours prior to transfection, GT1-7 cells were plated using coated 24 well plates (Nunc) at a concentration of 150,000 cells/well. Cells were transfected using Polyjet transfection reagent using manufacturers protocol. Approximately 7 hours post transfection, GT1-7 cells were switched to DMEM containing 0.1% BSA. Cells were either untreated or treated for 22 h with 1/8,000 DMSO or 100 nM TPA in DMSO. Approximately 36 hours post transfection; cells were washed with 1 ml of 1x cold PBS, and lysed with 55 μ l of lysis buffer (8.5 mM KH_2PO_4 , 91.5 mM K_2HPO_4 , 0.2% Triton X-100). Twenty μ l cell lysate was used to measure luciferase activity using a microplate luminometer with luciferase assay buffer (0.25 M Tris pH 7.8, 1 M MgSO_4 , 0.25 M ATP, luciferin). Similarly, β -galactosidase activity was measured using 70 μ l of Galacton per well. Transfections were done in triplicates and repeated in at least 4 independent experiments.

III. Results

Vax1 and cFos regulation of the GnRH promoter

We showed in the introduction that cFos, a known regulator of GnRH transcription, is induced within the same time frame as Vax1 repressed GnRH transcription (Illustration 5, Illustration 6). To determine if cFos and Vax1 coexist to repress GnRH transcription (Illustration 7), we studied the effect of AP1 site mutations on the GnRH E1/P luciferase promoter.

To validate cFos binding to the studied sites in the GnRH E1/P we initially confirmed the role these AP1 sites had in TPA induced repression of these promoter constructs. TPA, a protein kinase C activator, strongly increases cFos in immortalized GnRH neurons (14, 16), leading to repression of the GnRH E1/P (Figure 9, E/P).

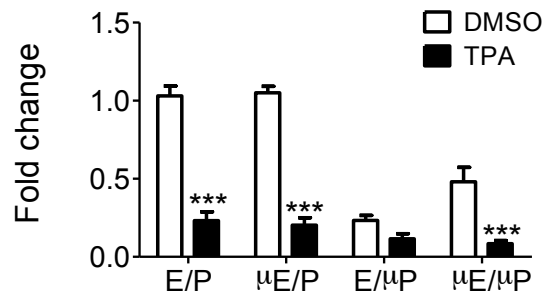


Figure 9. TPA induced cFos repression of the GnRH E1/P luciferase promoter

When treated with TPA 100 nM, GnRH transcription levels decreased significantly. μ=mutation

AP1 site of either E (enhancer 1), P (promoter), or both.

The constructs include mutations in either the enhancer, promoter or both of the GnRH E1/P driving luciferase. E/P indicates the WT model, with no mutations in either E1 or P. E/ μ P indicates a mutation in the promoter region of the GnRH E1/P. μ E/P indicates mutation in the enhancer region, and μ E/ μ P contains a mutated enhancer and promoter regions. Mutation of the AP1 site in the GnRH enhancer 1 (GnRH μ E/P) did not affect baseline transcription of this construct as compared to GnRH E1/P (Figure 9, lane E/P, μ E/P). It is surprising to see that baseline GnRH transcription decreases when the binding site for a repressor is mutated (Figure 9, lane E/ μ P). In the WT construct, GnRH E1/P transcription is reduced significantly by TPA, as expected (Figure 9, lane E/P compare white and black bar). Similarly, mutations of AP1 sites in just the enhancer as well as both enhancer and promoter display a similar effect on transcription (Figure 9, lane μ E/P and μ E/ μ P, compare white and black bar). However, mutation of AP1 in the promoter relieves, to some extent, TPA repression of the promoter, indicating the relative importance of the promoter region on TPA induced cFos regulation of GnRH transcription through the P region (Figure 9, lane E/ μ P compare white and black bar). The % effect of TPA on the different GnRH E1/P luciferase constructs with AP1 mutations are summarized in Figure 10, showing that mutation of AP1 in GnRH E/ μ P relieves to some extent TPA repressed transcription.

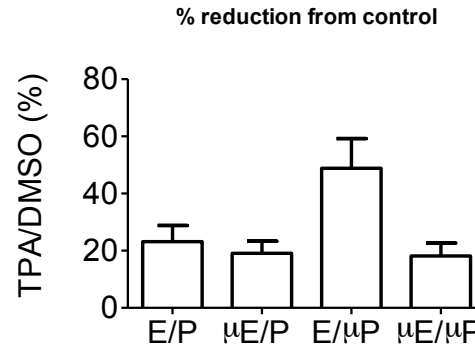


Figure 10. Percentage repression by TPA on the different AP1 mutation sites in the GnRH

E1/P

We know that cFos is able to repress GnRH transcription. However, we also wanted to know how basal cFos levels affects GnRH transcription and Vax1 regulation of the GnRH promoter. To do this, we treated cells with AFos, a dominant negative of cFos that acts to reduce cFos levels (17). Site directed mutations of AP1 sites in either the enhancer or promoter region of the GnRH E1/P driving luciferase were constructed to determine the role of cFos in Vax1 regulation of the GnRH promoter (this is the same mutations as used in Figure 9). In the WT construct, GnRH E1/P luciferase transcription is increased with the addition of AFos, as expected (Figure 11). A significant reduction of GnRH E1/P transcription by Vax1 was lost in the presence of AFos, suggesting basal cFos might have a minor role in Vax1 regulation of GnRH transcription (Figure 11). When the AP1 site was mutated in the GnRH promoter (GnRH E/μP), GnRH transcription was retained in the presence and absence of AFos, however repression of the control with Vax1 was increased (Figure 12). When the AP1 enhancer site was mutated (μE/P), Vax1 retained its capacity to repress in the presence of AFos (Figure 13). Lastly, when both enhancer and promoter regions of the AP1 site were mutated, GnRH transcription was unaffected

by Vax1, but in the presence of AFos, Vax1 regained its capacity to repress transcription (Figure 14). This suggests that cFos somehow interferes with Vax1 regulation of GnRH transcription.

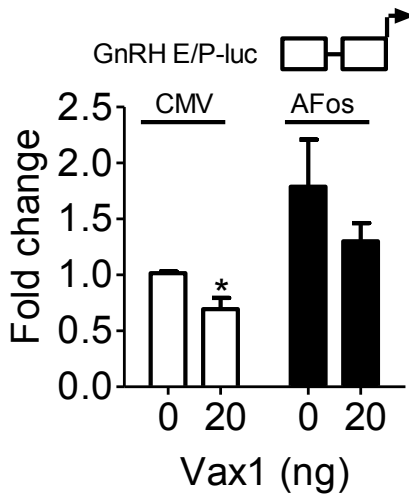


Figure 11. Effect of AFos and Vax1 in the GnRH E1/P-luciferase

Construct of GnRH E1/P-luciferase with 100 ng of AFos, and its empty vector 100 ng CMV, comparing fold change of GnRH transcription using either 0 ng or 20 ng of Vax1. Two-way

ANOVA followed by a Bonferroni test. * $p < 0.05$.

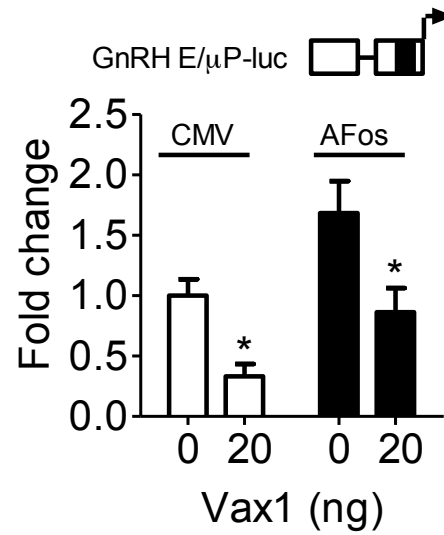


Figure 12. Effect of AFos and Vax1 in the GnRH E1/P-luciferase with AP1 mutation in the promoter

Construct of GnRH E1/P-luciferase with AP1 mutation in the promoter region with AFos, and its empty vector CMV, comparing fold change of GnRH transcription using Vax1 (Vax1 20) or its empty vector, pCMV6 (Vax1 0). Two-way ANOVA followed by a Bonferroni test. * $p < 0.05$.

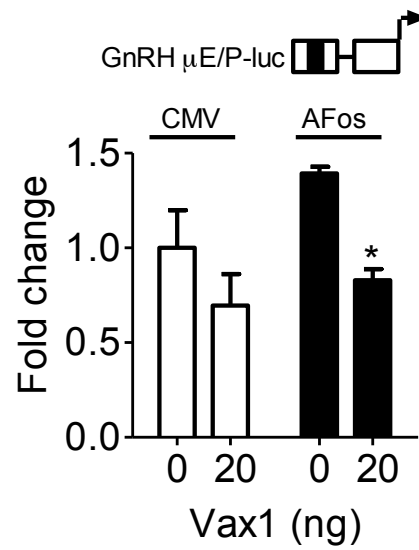


Figure 13. Effect of AFos and Vax1 on the GnRH E1/P-luciferase with AP1 mutation in enhancer 1

Construct of GnRH E1/P-luciferase with AP1 mutation in the enhancer 1 region with AFos, and its empty vector CMV, comparing fold change of GnRH transcription using either 0 ng or 20 ng of Vax1. Two-way ANOVA followed by a Bonferroni test. * $p < 0.05$.

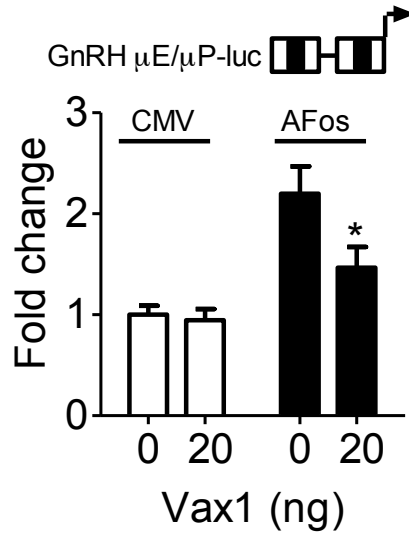


Figure 14. Effect of AFos and Vax1 on the GnRH E1/P-luciferase with AP1 mutation in the promoter and enhancer 1

Construct of GnRH E1/P-luciferase with AP1 mutation in the promoter and enhancer 1 region with AFos, and its empty vector CMV, comparing fold change of GnRH transcription using either 0 ng or 20 ng of Vax1. Two-way ANOVA identified a significant difference $p < 0.05$ of the effect of Vax1 on the different constructs.

Vax1 regulates immediate early gene cFos

Initially, we had observed that Vax1 overexpression could increase cFos transcription (Illustration 6). To determine if this association was a direct or indirect mechanism, we conducted luciferase assays on the cFos promoter. Our results indicated that Vax1 required the ATTA -59 bp site in the cFos promoter (not shown), but not the ATTA -313 bp site to increase cFos driven transcription (not shown). To confirm that Vax1 bound directly to the cFos promoter, we performed EMSA to reveal the level of interaction between Vax1 and the -59 bp and -313 sites in

the cFos promoter. None of the commercially available Vax1 antibodies tested so far have proven valid for EMSAs. Therefore we used the easy to transfect Cos cells and transfected them with Vax1-flag plasmid or its empty vector, pcmv6. Nuclear extract from these cells were used to determine specific Vax1 binding sites in the GnRH (control) and cFos promoters. We performed an EMSA with radioactive labeled probes to determine DNA binding. Cos cells transfected with a Vax1-myc overexpression vector expressed high levels of Vax1-flag protein which was as expected localized in the nuclear extract of the cell lysate (Figure 15).

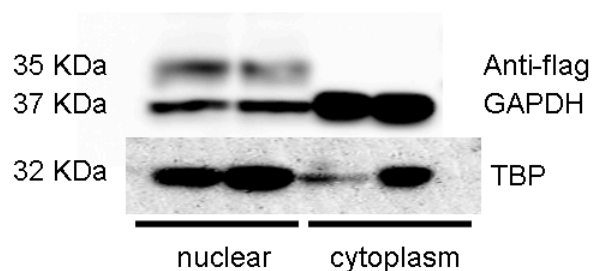


Figure 15. Vax1-flag is expressed in the nucleus

Westernblot, The nuclear localized TBP (TATA binding protein) was used to verify nuclear enrichment in the nuclear fraction. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used to confirm the cytoplasmic enriched fraction, and a flag antibody (flag) detected the Vax1-flag protein.

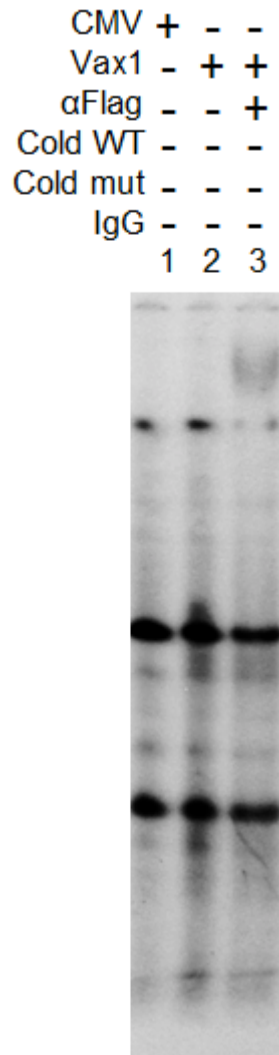


Figure 16. Vax1 binds to site -41 bp in the GnRH promoter

Supershift positive control, we confirmed Vax1 binding to GnRH promoter. EMSA was done to detect DNA binding at site -41 bp in the GnRH promoter. Lane 1 represents Vax1-flag empty vector called CMV. Lane 2 represents only the addition of Vax1-flag. Lane 3 represents the addition of Vax1 and antibody. The shift (red arrowhead) present in lane 3 confirms direct binding of Vax1 to this site of the GnRH promoter.

	Cos						Cos + GT1-7					
CMV	+	-	-	-	-	-	+	-	-	-	-	-
Vax1	-	+	+	+	+	+	-	+	+	+	+	+
α Flag	-	-	+	-	-	-	-	-	+	-	-	-
Cold WT	-	-	-	-	+	-	-	-	-	-	+	-
Cold mut	-	-	-	-	-	+	-	-	-	-	-	+
IgG	-	-	-	+	-	-	-	-	-	+	-	-
	1	2	3	4	5	6	7	8	9	10	11	12

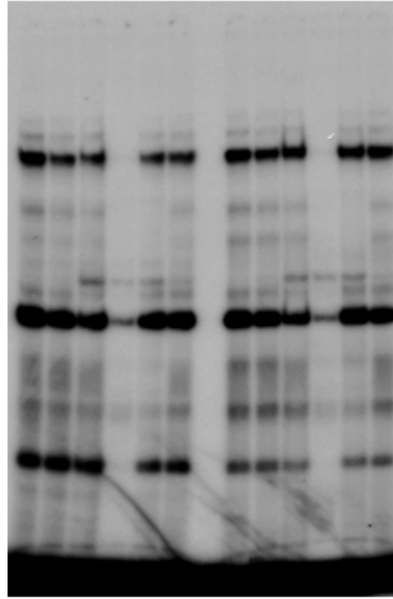


Figure 17. Vax1 does not bind directly to the -53 bp site in the cFos promoter

EMSA showing lack of binding of Vax1 to the cFos promoter -53 bp. Lane 1-7 using Cos cells: empty vector CMV, lane 2-6 and 8-12: Vax1-myc, lane 3,9: addition of anti-flag antibody, lane 4-6 and 10-12 controls with cold WT and cold mutant probes. Lanes 7-12 similar reactions using Cos+GT1-7 cells nuclear extracts.

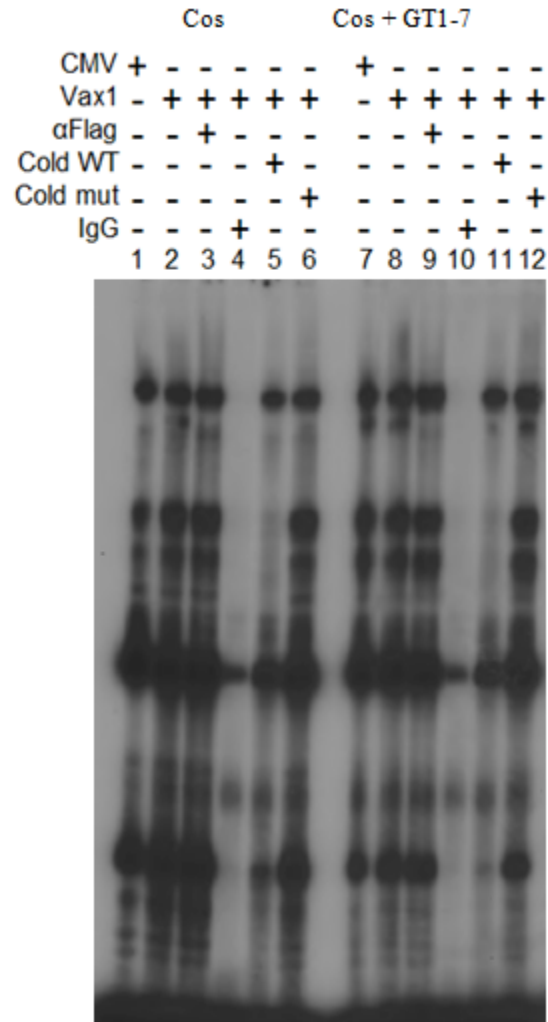


Figure 18. Vax1 does not bind directly to the -313 bp site in the cFos promoter

EMSA showing lack of binding of Vax1 to the cFos promoter -313 bp. Lane 1-7 using Cos cells: empty vector CMV, lane 2-6 and 8-12: Vax1-myc, lane 3,9: addition of anti-flag antibody, lane 4-6 and 10-12 controls with cold WT and cold mutant probes. Lanes 7-12 similar reactions using Cos+GT1-7 cells nuclear extracts.

Two sites (cFos -51 bp and cFos -313 bp) were analyzed for Vax1 binding potential in the cFos promoter. Since it has been previously shown in the lab that Vax1 binds to the -41 bp

GnRH promoter (Figure 16), the GnRH promoter -41 bp site served as a positive control for Vax1-flag antibody supershift, and the cFos promoter -313 bp served as a negative control as we did not expect to see a shift occur on that site. Conditions as shown in Figure 16 determined Vax1 binding in the GnRH promoter -41 bp site as shown by the supershift in lane 3. However, the lack of shift observed in cFos -51 indicates that Vax1 does not bind cFos directly through this site in Cos cells. As no supershift was seen on the -59 bp site in Cos cells, we speculated that maybe GT1-7 cells contain proteins that Vax1 interact with that were necessary for Vax1 binding to this site. To test this hypothesis we added GT1-7 nuclear extracts to the Cos cell nuclear extracts transfected with pcmv6 or Vax1-myc. No supershift was observed in the presence of GT1-7 cell nuclear extract on either the -59 bp cFos site (Figure 17), nor on the -313 bp site (Figure 18).

IV. Discussion

Reproductive success is based on a series of molecular models driving transcription of several important regulatory genes such as GnRH. In this chapter we studied more in detail how Vax1 regulated the GnRH promoter in the presence and absence of the immediate early gene cFos.

Confirmation of an AP1 site in the GnRH promoter where cFos binds to regulate GnRH transcription

To determine the role of AP1 sites in GnRH regulation, site mutations on either the enhancer or promoter regions were studied. We treated the cells using TPA, a known inducer of cFos, to determine the effect of GnRH E1/P luciferase promoter activation when AP1 sites were mutated (15). TPA induces cFos via PKC activation, thus repressing GnRH transcription in GT1-7 cells. We have seen that in the presence of the AP1 mutation in the GnRH promoter, the effect

of TPA is decreased, indicating an important role of the studied AP1 site in the regulation by cFos of GnRH transcription. Interestingly, when both the GnRH enhancer and promoter regions were mutated (AP1 mutations), TPA regained its ability to repress GnRH transcription. This data indicates that certain interactions via the promoter region of the cFos promoter was sufficient to allow GnRH repression.

Eliminating basal cFos by the use of AFos increase GnRH transcription

Afos, a dominant negative of cFos, removes basal cFos levels in the cell (17). This allowed us to determine whether or not basal levels of cFos regulated GnRH transcription. Our data show that with the addition of Afos, GnRH transcription increased dramatically. Since cFos is a known repressor of the GnRH promoter, increased GnRH transcriptional activity in AFos transfected cells was expected.

Mutations of AP1 sites in the GnRH promoter affects Vax1 regulation of the GnRH promoter

Mutations of AP1 sites in the GnRH E1/P changes the capacity of Vax1 repression of GnRH transcription. We know that Vax1 is expressed in GnRH neurons and can regulate the GnRH promoter directly (see introduction). From our data, we see that Vax1 loses its ability to repress GnRH transcription with both AP1 mutations of the enhancer and promoter regions, deeming AP1 necessary for Vax1 regulated GnRH transcription (Figure 19).

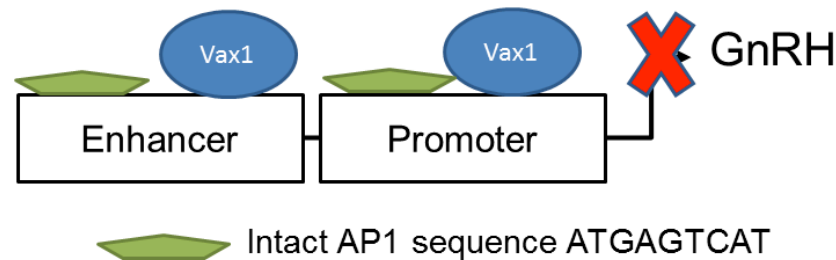


Figure 19. Vax1 repression of the GnRH promoter requires intact AP1 sites in close proximity to its binding sites (see also Illustration 8)

AFos and Vax1 co-transfections on the GnRH E1/P with AP1 mutations

AP1 mutations in either the GnRH E1 and/or P regions of the GnRH promoter altered Vax1's ability to control GnRH repression. Mutation of AP1 site in the GnRH promoter region retained GnRH transcription in the presence and absence of cFos. However, repression of the GnRH E/ μ P with Vax1 was not seen in previous experiments (not shown). We will confirm this effect with different Vax1 concentrations in future experiments. However, concomitant mutation of AP1 sites in GnRH enhancer and promoter regions abolishes Vax1 capacity to regulate GnRH transcription. This repression was restored with the removal of cFos via AFos. This indicates a very complex interaction in the control of GnRH transcription between cFos and Vax1. Future experiments will be designed to determine exactly how Vax1 and cFos interfere with each other to regulate GnRH transcription (Figure 20).

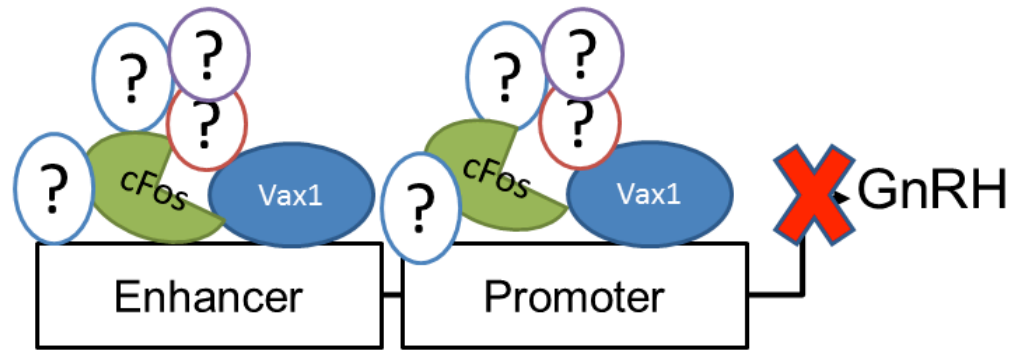


Figure 20. Hypothetical model of possible Vax1 and cFos interaction requiring additional proteins in the transcriptional complex regulating GnRH transcription

Vax1 regulation of the cFos promoter

We had previously determined that Vax1 required the -59 bp ATTA site in the cFos promoter by luciferase assays (not shown). However, none of the numerous bands seen on the EMSA of the cFos promoter oligos represented Vax1 binding and it remains unknown what transcription factors binds the studied constructs. We will in future experiments attempt to identify who is binding to the studied sites. Thus, Vax1 either binds to other sites in the cFos promoter or its action might be indirect (Figure 21).

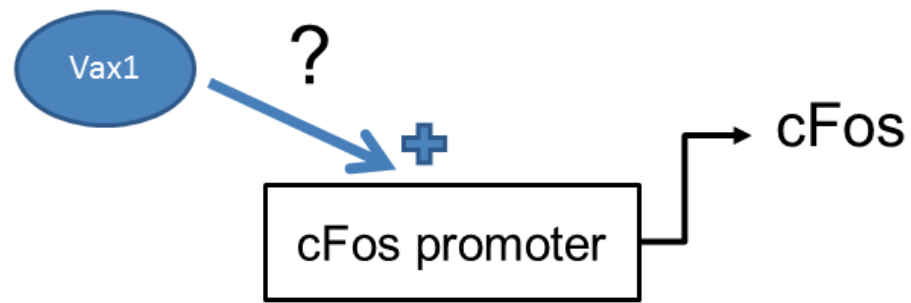


Figure 21. Vax1 increases cFos transcription through an unknown mechanism

General Conclusions

GnRH neurons regulate the pituitary gland to release subsequent hormones, LH and FSH, which activate the release of sex hormones (Illustration 1). Current studies have shown that there is a significant reduction of GnRH neurons in Vax1 HET mice (unpublished data from the lab), indicating an obstruction of GnRH migration from the olfactory placode to the hypothalamus, where mature GnRH neurons are localized. We have determined that Vax1 expression during development is required for GnRH neurons to reach the hypothalamus, thus concluding Vax1 as a potential candidate in IHH patients. It is believed that numerous cases of IHH are due to composite gene mutations that together lead to complete infertility (Bianco and Kaiser 2009). Based on our fertility study in chapter 1 we propose that Vax1 is a potential candidate in composite IHH. Interestingly, Vax1 heterozygote mice showed various subfertility characteristics including smaller litter size and fewer litters. In females, estrous cycling seemed to be prolonged as the heterozygous mice remained in the metestrus phase for a longer time period than their WT counterparts. Additionally, less corpora lutea suggested defects in the signaling of the HPG axis. In males, decreased sperm quality and sperm count was observed. Thus, both heterozygote males and females showed evidence of abnormal reproduction.

In chapter 2, we were studying the regulation of the GnRH promoter. Our goal was to determine if Vax1 could directly regulate the GnRH promoter. Due to the length of the transfection experiments (the cells are transfected for approximately 7h, followed by a 22 h period where the plasmid is transcribed and the protein is translated), it was possible that Vax1 could both act directly on the GnRH promoter as well as induce an immediate early gene whom would act in parallel with Vax1 regulating GnRH transcription. Our data indicate that during this time frame Vax1 directly repressed GnRH transcription as well as induced cFos, who at its turn repressed the GnRH promoter (Illustration 7). Our data supports, that the hypothetical model 1

(Illustration 7), that Vax1 regulates both the GnRH and cFos promoters. Although we are currently unable to explain our data completely, we have here shown evidence that by removing basal AFos, Vax1 does not repress the GnRH promoter as efficiently as in the presence of basal cFos (Figure 11). In addition, the concomitant mutation of AP1 sites in the GnRH E and P abolished Vax1 regulation of this construct (Figure 14, Figure 19). And removing basal cFos can in some cases reestablish Vax1 repression of the GnRH promoter (Figure 14). Taken together this shows a complete unexpected and possible novel interaction between a homeodomain binding protein (Vax1) and an immediate early gene (cFos). This surprising interaction is extremely interesting as it is a way of means for the cell to rapidly change the capacity of a transcription factor to regulate gene transcription, without having to degrade the protein in question. Future studies will aim at dissecting the complex transcription regulation mechanism, which might prove to involve a number of here unidentified proteins integrating the transcriptional complex allowing this interesting transcription interaction (Figure 20).

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