UNIVERSITY OF CALIFORNIA SAN DIEGO

A Patient Mutation in DLX1 Disrupts Homeodomain Binding and Gonadotropin-Releasing Hormone Expression in GnRH Neurons

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in

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by

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2019

The Thesis of Austin Yung Tzen Chin is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

2019

Dedication

I would like to dedicate this thesis to:

my late brother, Andrew. Thank you for continually inspiring me to keep pushing, even when times get tough.

Table of Contents

List of Figures and Tables

Abbreviations

AVPV	Anteroventral Periventricular Nucleus
ARC	Arcuate Nucleus
DLX1	Distal-less homeobox 1
DLX1(c.ins381A)	DLX1 mutation, insertion of adenine at 381th base pair
DLX1-HA	DLX1 with an 3x HA tag in the N-terminus
DLX1(c.ins381A)-HA	DLX1(c.ins381A) with an 3x HA tag in the N-terminus
DN	Dominant negative
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
HI	Haploinsufficiency
ATTA-luc	Homeodomain binding site-driven luciferase reporter plasmid
HPG axis	Hypothalamic-Pituitary-Gonadal axis
IGD	Isolated Gonadotropin-Releasing Hormone Deficiency
LH	Luteinizing hormone
minTK	Minimal thymidine kinase plasmid backbone
NMD	Nonsense Mediated Decay
POA	Preoptic Area
RSV	Rare Sequence Variant
TKβgal	Thymidine kinase promoter-driven βgal expression plasmid
VNO	Vomeral Nasal Organ
β-gal	β-galactosidase

Acknowledgements

I would like to acknowledge my post-doctoral mentor Dr. Jessica Cassin and the chair of my committee Dr. Pamela Mellon. Their guidance through my time as an undergraduate and as a master's student has made all this possible and I cannot thank them enough for the unparalleled patience and support.

Abstract of the Thesis

A Patient Mutation in DLX1 disrupts Homeodomain Binding and Gonadotropin-Releasing Hormone Expression in GnRH Neurons

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Puberty and fertility are mediated by the regulation of Gonadotropin-Releasing Hormone (GnRH). Low levels of GnRH can result in abnormal puberty and infertility, which are characteristics of a condition known as Isolated GnRH Deficiency (IGD). The etiology of IGD has yet to be fully elucidated, but recently the reproductive sciences have taken a genetics-based approach towards understanding how IGD affects the GnRH neurons, and more broadly, the reproductive axis. An exome-wide association study has identified a patient mutation in DLX1 to

be associated with IGD. To characterize functional consequences of this mutation of DLX1 *in vitro*, we developed a patient mutation model in mature and developing immortalized GnRH neuron cell lines. We observed that the IGD patient mutation of an insertion of an adenine at the 381th base pair of the DLX1 gene leads to impaired homeodomain binding site activation and lower GnRH expression. These data lay the foundation for future study of rare genetic variants that are associated with IGD and the mechanisms responsible for IGD and Kallman's syndrome etiology.

Introduction

Fertility and the HPG axis

About 15% of couples suffer from impaired fertility in the United States [1]. However, the reasons for impaired fertility vary case by case. Causes of infertility may range from abnormal puberty to impaired hormone regulation that occurs at any age after puberty. Puberty and fertility are hormonally regulated by the three major endocrine centers that form the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1). Regulation of hypothalamic, pituitary, and gonadal secretions is essential to initiating puberty and maintaining fertility. The hypothalamus contains specialized neurons that secrete gonadotropin-releasing hormone (GnRH), which stimulates downstream events of the HPG axis, including gonadal germ cell maturation and secondary sex characteristics [2,3]. Ultimately, regulation of the HPG axis and GnRH protein are responsible for sexual maturation and the ability to conceive [4].

Function of the HPG axis begins principally in the hypothalamus, where the protein GnRH is synthesized and delivered to the anterior pituitary. GnRH stimulates the gonadotrope cells of the pituitary to secrete gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Fig. 1) [5]. LH and FSH stimulate the gonads to secrete sex steroids that will provide negative feedback to the hypothalamus via kisspeptin neurons [6]. There are two populations of kisspeptin neurons, located in the hypothalamus [6]. Specifically, the kisspeptin neuron populations are located in the regions of the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC). AVPV and ARC kisspeptin neurons differentially mediate the release of GnRH through increasing or decreasing kisspeptin release, respectively [7-11].

ARC kisspeptin neurons regulate the rhythmicity of GnRH release and are negatively regulated by sex steroid feedback [6]. AVPV kisspeptin neurons are associated with ovulation and a strong, singular surge of luteinizing hormone; these neurons are positively stimulated by high concentrations of sex steroids [6]. Figure 1 depicts the kisspeptin neurons' differential regulation of GnRH and subsequent action on the rest of the Hypothalamic-Pituitary-Gonadal (HPG) axis. If endocrine communication is altered at any level of the HPG axis, an individual can be infertile. In humans, diagnosis of infertility is commonly determined by low sex steroids in a hormone test, since a decrease in GnRH or the gonadotropins ultimately results in low sex steroids [12].

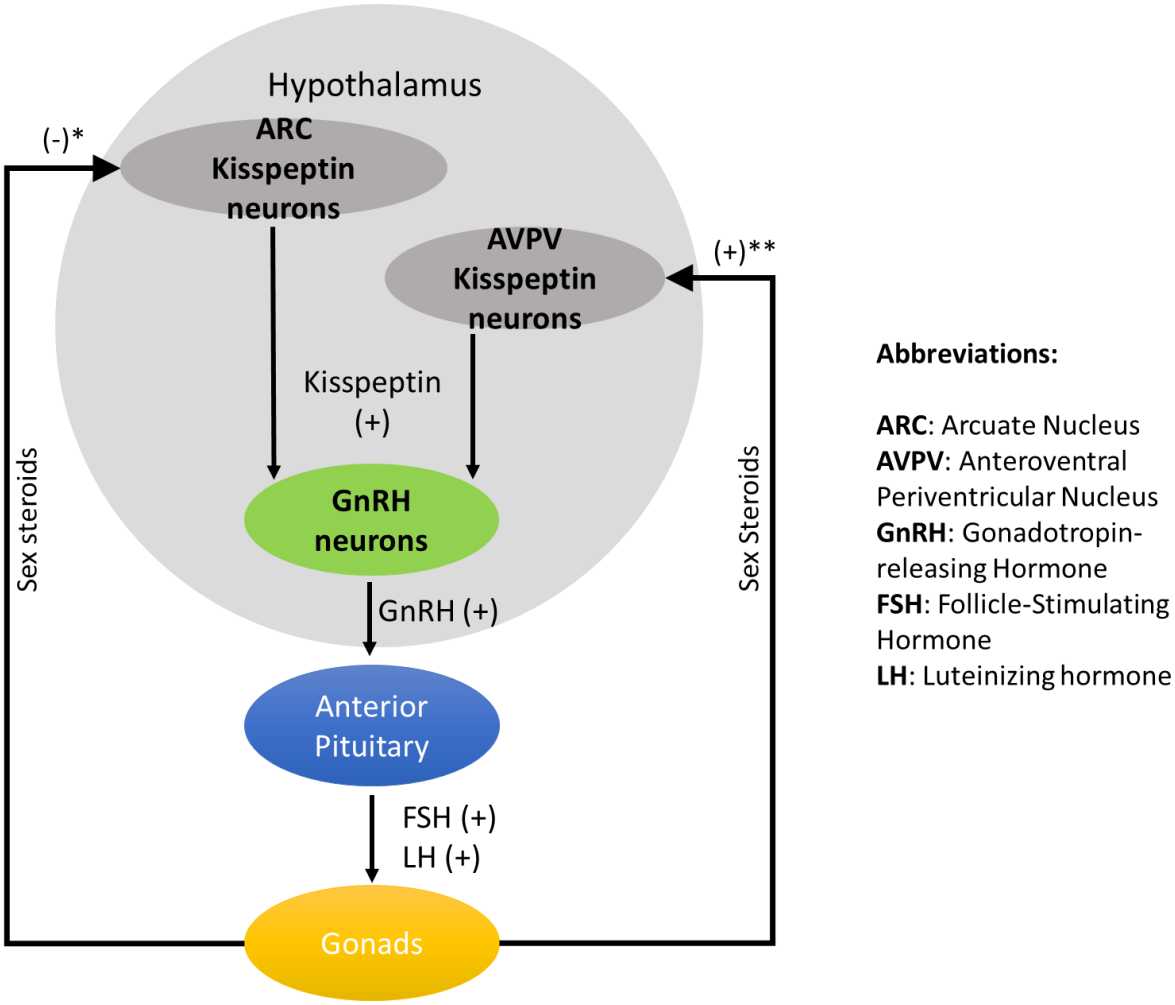

Figure 1. The Hypothalamic-Pituitary-Gonadal Axis. GnRH neurons secrete GnRH protein that stimulates the anterior pituitary. The anterior pituitary secretes LH and FSH to stimulate the gonads to secrete sex steroids. Depending on the concentration of systemic androgens in males or estrogens in female, two populations of kisspeptin neurons will differentially regulate the release of kisspeptin, a positive regulator of GnRH release. *ARC kisspeptin neuron secretion of kisspeptin is inhibited by low to medium concentrations of sex steroids. **AVPV kisspeptin neuron secretion of kisspeptin is activated, but only at high concentrations of sex steroids.

GnRH Neuron Migration

During embryonic development, GnRH neurons migrate from the olfactory placode to the preoptic area by birth (Fig. 2) [13,14]. Once in position, GnRH neurons can properly secrete GnRH to the anterior pituitary through axonal projections to the median eminence [15]. The precise mechanism that drives GnRH neuron migration and the inputs to the GnRH secretory system that initiate puberty are an area of ongoing research.

One type of infertility is Isolated Gonadotropin-Releasing Hormone Deficiency (IGD), which is caused by abnormal GnRH levels that result in an individual's infertility and abnormal puberty [16]. Furthermore, a subtype of IGD is a disease of neuron migration known as Kallman's syndrome [17]. A key phenotype of model animals for Kallman's Syndrome and symptom of Kallman's patients is anosmia, or the inability to smell, which often coincides with impaired neuron migration in mice [18, 19]. In humans, anosmia is a strong determinant to distinguish Kallman's syndrome from normosmic IGD, but bona fide diagnosis of Kallman's syndrome can only be done post-mortem, as mis-migration of GnRH neurons is the true determinant and can only be confirmed by autopsy. The impaired fertility and sexual maturation experienced by Kallman's patients is assumed to arise, at least in part, from impaired GnRH neuron migration [20]. Further research on the factors responsible for GnRH neuron migration will benefit current knowledge of the neuron migration phenomenon in general. As migration is part of GnRH neuron maturation, a deeper understanding of GnRH neuron maturation could provide a target of therapy for abnormal puberty, IGD, and Kallman's syndrome.

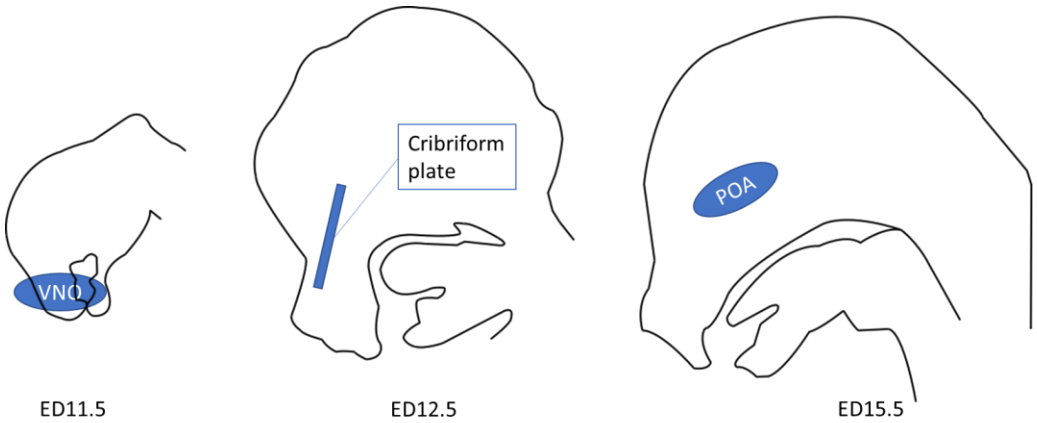

Figure 2. GnRH Neuron Migration in Mouse Embryos. Schematic of developing GnRH neuron locations in sagittal head sections from embryonic mice at embryonic days (ED) 11.5, 12.5, 15.5. GnRH neurons migrate from the Vomeral Nasal Organ (VNO) across the cribriform plate, to the Pre-Optic Area (POA). Adapted from "The Homeodomain Proteins SIX3 and SIX6 Are Essential in The Development, Survival, and Migration of GnRH Neurons." [14] modified with permission.

Genetics and Infertility

The reproductive sciences have taken a genetics-based approach toward elucidating the cellular and molecular mechanisms that drive the Hypothalamic-Pituitary-Gonadal (HPG) axis to better understand infertility, IGD, and Kallman's syndrome. Roughly 30 genes have been identified as causative for IGD [21]. Many genes on this list include genes that are associated with the classical HPG axis mechanism, such as genes coding GnRH, the GnRH receptor, Kisspeptin, or the Kisspeptin receptor [21]. Another notable category of genes on the list of IGD-causative genes are genes associated with embryonic development, neuron migration, and neuron maturation [21].

A collaboration has been formed between the Crowley lab and the Mellon lab, whereby the Crowley lab identifies rare sequence variants (RSVs) that are associated with IGD in human patients, and the Mellon lab will investigate the consequences of the RSVs in cell culture models within the context of specific neuron populations to better understand how those genes and their RSVs interact with the HPG axis and fertility. Collaborators from the Crowley lab performed Whole Exome Sequencing (WES) of IGD patients to identify novel disease-associated mutations. The Crowley Lab's bioinformatics workflow is principally designed to isolate *de novo* mutations in patients that could be causative to IGD. With databases for exomes, they isolate candidate rare sequence variants (RSVs) that are likely associated with the disease. By applying constraints for loss of function, missense, and regional mutations, the Crowley lab further filters candidate RSVs for pathogenic implications. Pathway analysis, literature searches, and common trait analysis are used to determine the RSVs potential relationship to disease phenotypes.

To study the isolated RSVs in individual cell types, the Mellon lab uses murine-derived immortalized hypothalamic cell lines. Representing the hypothalamus, we can utilize the GN11, GT1-7, KTaV, and KTaR cell lines. The GN11 and GT1-7 cell lines are both derived from GnRH neurons, but GN11 cells were found to mimic developing neurons whereas the GT1-7 cells are derived from mature neurons; both lines express GnRH and secrete the corresponding protein at physiologically relevant levels corresponding to the neurons from which they are derived [22,23]. Similarly, the KTaV and KTaR cell lines express kisspeptin at levels that correspond to their *in vivo* AVPV and ARC counterparts at physiologically relevant levels [24]. Thus, the KTaV and KTaR cell lines serve as models for changes in hormone expression. Because GN11 cells were designed from developing GnRH neurons, they can migrate *in vitro* [23]. Thus, the GN11 cell line is a fitting model to study forms of IGD that arise due to developmental disorders, specifically the effects of gene expression changes on GnRH neuron development.

Recently, the Crowley lab has found a proband mutation in the gene DLX1 in the WES study. Since the RSV has been identified, the Mellon lab can now begin deeper investigation of DLX1 and its mutant allele, DLX1(c.ins381A).

The Role of DLX1 in Fertility

Distal-Less Homeobox (DLX) genes are antennapedia-class homeobox genes, which are involved in determining pattern formation in the embryo. The Mellon lab has previously identified genes from the DLX gene family as essential to GnRH neuron migration and fertility [27]. DLX1, as a transcription factor, binds to homeodomain ATTA sites in DNA to regulate the rate of transcription of other genes [25]. Specifically, DLX1 regulates craniofacial structures in

the anterior forebrain [26]. The Mellon lab has previously shown that DLX1 binds to ATTA sites in the GnRH enhancer and promoter (e/p) and that overexpression of DLX1 in hypothalamic cell lines enhances GnRH activation [27]. Whole-body knockout mice lacking *Dlx1&2* had mis-migrated GnRH neurons, impaired sexual maturation, and were infertile [27]. In wildtype mice, *Dlx1* is expressed in GnRH neurons during embryonic development [27]. These data show that proper expression of DLX1 is required for normal GnRH neuron development, GnRH secretion, and fertility.

Investigating how DLX1(c.ins381A) affects Fertility

The proband was found to be heterozygous for wildtype DLX1 and an allele with a mutation, DLX1(c.ins381A). The mutant allele contains an insertion of an adenine after the 381th base pair of the sequence for DLX1. *In silico*, this introduces a premature termination codon (PTC) that prevents translation of the homeodomain-binding region and C terminus of the protein [28]. For this reason, we have focused our studies on the functional ability of the DLX1(c.ins381A) mutant protein. Although the role of DLX1 has been studied in the reproductive axis, nothing is known about the mutation DLX1(C.ins381A) or its effects on the HPG axis. This current study covers functional analysis of DLX1(c.ins381A) to better understand the etiology IGD and Kallman's Syndrome. This project will build the foundation for understanding this rare mutation in DLX1 and future treatment for infertility due to IGD or Kallman's Syndrome. This study sets the precedent for future studies involving other mutations that our collaborators identify.

Materials and Methods

Cell Culture

The KTaR and KTaV cell lines were kindly provided by Dr. Patrick Chappell [24]. The GN11 cell line was kindly provided by Dr. Sally Radovick [29]. NIH3T3 [30], GT1-7 [22], GN11, KTaR, and KTaV cells were cultured in complete media, containing DMEM (Mediatech Inc.), containing, 10 % fetal bovine serum (Omega Scientific, Inc), and 1x penicillin-streptomycin (Hyclone Laboratories) in a humidified 5% CO2 incubator at 37°C. Cells used were between passages 8 and 29.

Plasmid Constructs

DLX1 expression plasmid in a pcDNA3.1+ backbone was ordered from Addgene. DLX1(c.ins381A) expression plasmid was then made from the DLX1 expression plasmid using Q5 mutagenesis (New England Biolabs). For western blots, immunocytochemistry, and luciferase assays, we used Q5 mutagenesis to add a 3x HA tag at the N terminus of the DLX1 and DLX1(c.ins381A) expression plasmids (New England Biolabs).

In both luciferase assays, the DLX1 and DLX1(c.ins381A) expression vectors were driven by a constitutive promoter, CMV, in a pcDNA3.1+ backbone. An empty vector for pcDNA3.1+ was used as a transfection control. Transfection efficiency was normalized by using an expression vector for β-galactosidase (β-gal) driven by the thymidine kinase promoter (TKβgal), calculating a luciferase/β-galactosidase (luc/β-gal) value. Because the experiments were designed to characterize the heterozygote, we transfected cells with equal parts DLX1 and DLX1(c.ins381A) expression plasmids and compared data to conditions that model homozygotes: containing equal parts DLX1 or DLX1(c.ins381A) expression plasmid and vehicle

control. Additionally, we included an all vehicle control condition to normalize to for fold change analysis.

For homeodomain binding luciferase assays, the ATTA-luciferase plasmid (ATTA-luc) contains 5x CAATTACA multimers from the rat GnRH promoter (positions -55 to -48 relative to the transcription start site) in a minimal thymidine kinase (minTK) backbone. The minTK promoter (positions -138 to -50 relative to the transcription start site) drives expression of the luciferase reporter. GT1-7, GN11, and NIH3T3 cells were transfected with 200 ng/well of either DLX1, DLX1(c.ins381A), or 200 ng of both and co-transfected with 200 ng/well ATTA-luc, and 100 ng/well TkβGal (-109 thymidine kinase promoter on βGal) expression vectors. For every ATTA-luc containing condition, there was an identical well that was transfected with a minTK-luciferase plasmid in place of the ATTA-luc. To normalize for background luciferase activity, luc/β-Gal for each ATTA-luc containing well was divided by that of its corresponding minTK containing well.

For GnRH enhancer/promoter (e/p) luciferase assays, GnRH e/p-luciferase plasmids (GnRH e/p-luc) contain the sequence for rat GnRH enhancer (positions -1571 to -1863 relative to the transcription start site) and GnRH minimal promoter (-173 to -112) upstream of the luciferase reporter gene in a PGL3 (Promega) backbone [32]. GT1-7 cells were transfected with 200 ng/well of either DLX1, DLX1(c.ins381A), or 200 ng/well of both and co-transfected with 400 ng GnRH e/p-luc, and 200 ng TKβGal expression vectors. PGL3-luciferase was used as a background luciferase activity control.

Luciferase Assays

For luciferase assays GN11 and NIH3T3 cells were seeded into 12-well plates (Nunc) at 20,000 and 30,000 cells per well, respectively. 24 hours after seeding, cells underwent transient transfection in triplicate using Polyjet (SigmaGen Laboratories) and serum-free DMEM. Transfection efficiency was normalized by expression of the TKβGal reporter. 24 hours after transfection, cell media was aspirated and replaced with complete media. 72 hours after transfection, cells were washed with Phosphate Buffered Saline (PBS) and collected in 60 μL lysis solution (8.5 mM KH_2PO_4, 91.5 mM K_2HPO_4, 0.2% Triton X-100). 25 μL of lysed cells were used to quantify Luciferase and β-galactosidase expression. β-galactosidase activity was measured by injecting 100 μL of Tropix Galacto-light β-galactosidase assay buffer (Applied Biosystems) into each sample and luminance was quantified one second after injection. Luciferase activity was measured by injecting 100 μL of luciferase assay buffer (25 mM Tris pH 7.4, 15 mM $MgSO_4$, 10 mM ATP, and 65 μM firefly D-luciferin) and luminance was quantified one second after injection. Both β-galactosidase and luciferase activity recordings were measured using a Veritas Microplate Luminometer (Turner Biosystems) in 96 well, flat bottom assay plates (Corning Inc.).

RT-qPCR

GT1-7, GN11, KTaR, KTaV, and NIH3T3 cells were plated in 10 cm plates (Nunc) and collected at 70% confluency. Total RNA from GT1-7, GN11, KTaR, KTaV, and NIH3T3 cells was extracted using TRIzol (Invitrogen). RNA was isolated with RNA Clean & Concentrator kit (ZYMO). DNA was removed with DNase (New England BioLabs), then isolated RNA underwent reverse transcription using iScript cDNA synthesis kit (Bio-Rad). cDNA was

quantified using iQ SYBR Green Supermix (Bio-Rad) on a Q-RT PCR iQ5 real-time detection system (Bio-Rad). Primers used for amplifying cDNA products are summarized in Table 2. Data were analyzed with the ΔΔCT method. DLX1 expression was normalized to the housekeeping gene H2AFZ. Data are presented as fold-change relative to DLX1/H2AFZ expression in NIH3T3 cells.

TABLE 1. qPCR Primers List

Gene	Forward Primer	Reverse Primer	Source
Dlx1	AAGAGTACGGTGGTGGAAGG	ATCTTGACCTGCGTCTGTGT	Custom Oligo (Integrated DNA Technologies)
H2afz	TCACCGCAGAGGTACTTGAG	TCTCCCGGCCCACCACGTAT	Hoffmann et al. 2017 [31]

Protein collection

For western blots, GN11 cells seeded in 10 cm plates (Nunc) were transfected with 6 µg of DLX1-HA or DLX1(c.ins381A)-HA expression vectors (Table 1) in Polyjet (SigmaGen) and serum-free media (MediaTech) at 70% confluence. 72 hours after transfection, they were scraped in 500 µL Pierce RIPA buffer (Thermo Scientific) and 1X Protease Inhibitor Cocktail (Sigma Aldrich) then left on ice to swell. Debris was separated by centrifugation at 14,000 x g for 10 minutes in 4°C. Protein concentration was determined using the Bio-Rad Protein Assay.

Western Blots

Protein samples were dissolved and denatured in loading buffer (62.5 mM Tris-HCl pH 6.8, 2.5% SDS, 0.002% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol). To optimize for protein concentration, we first ran 10 µL of whole cell extract, then adjusted protein sample input by western blot band intensity. Protein sample was separated by 4-20% acrylamide gradient (Bio-Rad) SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore) for one hour at 100V in 4°C. The membrane was washed in TBS 1% Tween 20 (TBS-T) and was blocked in 5% nonfat dry milk (Apex, Bioresearch Products, Genesee Scientific) in TBS-T. Primary antibodies were diluted in 3% milk in TBS-T and incubated overnight at 4°C with gentle agitation. Rabbit anti-HA (1:3000, Cell Signaling Technologies, CAT# 3724, LOT#9) was diluted in 3% milk in TBS-T and incubated overnight at 4°C with gentle agitation. Goat anti-rabbit (1:10000; Santa Cruz Biotechnology, CAT# sc-2004 LOT# G2111) horseradish peroxidase (HRP)-conjugated secondary antibody was diluted in 3% milk in TBST and incubated at room temperature with gentle agitation for 1 hour. Antibody binding for anti-HA was detected by SuperSignal West Dura Extended Duration Substrate and

for anti-βActin, we used SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific). Loading normalization was performed using HRP-conjugated primary antibodies for mouse anti-βActin (1:20,000; Abcam, CAT#ab8227, LOT#GR276781-11). Image visualization and quantification was performed using ImageJ (NIH).

Immunocytochemistry

GN11 cells were seeded in 24 well plates (Nunc) at 10,000 cells per well and transfected with 0.2 µg of DLX1-HA or DLX1(c.ins381A) expression plasmid (Table 1) with Polyjet (SigmaGen) and serum-free media (MediaTech) per well. 24 hours after transfection, cell media was aspirated and replaced with complete media. 72 hours after transfection, cells were washed in PBS and fixed in 4% paraformaldehyde in PBS. Cells were blocked and permeabilized in 4% goat serum in PBST 30 minutes at room temperature.

Primary antibodies were diluted in PBST. The primary antibodies used was rabbit anti-HA (1:1000, Cell Signaling Technologies, LOT#9), Primary antibodies were incubated overnight at 4°C with gentle agitation. Secondary antibodies were diluted 1:1000 in PBST. The secondary antibody used was goat anti-rabbit conjugated to Cy3 (Abcam, ab97075, LOT#GR3211154-9). To label the nucleus, we stained cells with 570 nM DAPI diluted in PBS (Roche Diagnostics). Secondary antibodies were incubated for 1 hour at room temperature with gentle agitation. Cells were image using a ZOE Fluorescent Cell Imager (Bio-Rad). Image visualization was performed using ImageJ (NIH).

Results

DLX1 is Highly Expressed in the Developing GnRH Neuron Cell Line

Previous studies have shown that *Dlx1* is expressed in the hypothalamus of developing mouse embryos [25]. To further characterize the expression pattern in cultured cells, we screened different hypothalamic immortalized cell lines for *Dlx1* expression. Since Antennapedia class homeobox domain proteins are temporospatially expressed, we screened both developing GnRH neurons and maturing GnRH neurons, modelled by GN11 cells and GT1-7 cells, respectively. ARC and AVPV Kisspeptin neurons regulate GnRH and project onto GnRH neurons. Since *Dlx1* has been shown to be essential for GnRH expression, we screened both Kisspeptin neuron populations (KTaR and KTaV cell lines) as well. As a non-hypothalamic cell line control, we used NIH3T3 cells, derived from fibroblasts.

As shown in figure 3, only GN11 cells expressed more *Dlx1* than the non-hypothalamic control. n=1, so statistical analysis is not available with the current data available. As this is a current experiment, n is expected to increase while data continue to follow the observed trend. High *Dlx1* expression in the GN11 but not GT1-7 cells shows that *Dlx1* expression is different at different stages of GnRH neuron development. A mutation in a gene whose expression pattern is increased during development has strong developmental implications.

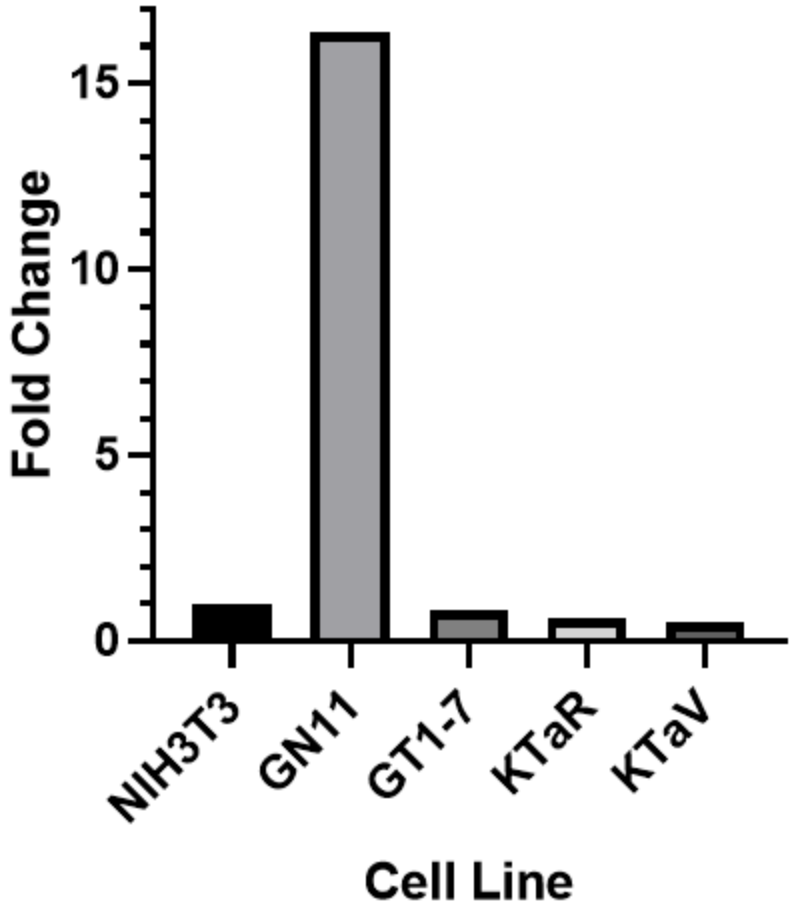

Figure 3. DLX1 is highly expressed in developing GnRH neurons *in vitro*. Murine-derived cell lines used: NIH3T3 (fibroblast), GN11 (developing GnRH Neurons), GT1-7 (mature GnRH Neurons), KTaR (ARC Kisspeptin Neurons), and KTaV (AVPV Kisspeptin neurons). Reverse Transcriptase Quantitative PCR. DLX1 transcripts were enriched in maturing GnRH neurons. Expression of DLX1 was normalized to expression of housekeeping gene H2AFZ. Relative fold change of expression was normalized to DLX1/H2AFZ expression in NIH3T3 cells. n=1. No statistical analysis possible currently.

DLX1(c.ins381) introduces a Premature Stop Codon that Results in a Truncated Protein

In silico analysis of the *Dlx1(c.ins381A)* sequences reveals that the insertion of an adenine after the 380th base pair introduces a frameshift in the open reading frame. The frameshift mutation causes an early stop codon in the coding region, otherwise known as a premature termination codon (PTC) [28]. Nonsense Mediated Decay (NMD) is a surveillance pathway that reduces errors in gene expression by degrading transcripts with PTCs. NMD is mediated by the ribosome and the exon junction complex (EJC). In translation under normal conditions, the ribosome passes the EJC, displacing it from the transcript, and stops at the termination codon [33]. When the ribosome passes a stop codon before the final EJC of a protein, it recruits the surveillance complex which degrades the transcript [33]. Thus, the order in which EJC and stop codon occur is the quality check that initiates NMD. NMD recognizes stop codons that greater than 50 nucleotides upstream of the final exon [33]. Because DLX1(c.ins381A)'s PTC occurs 124 nucleotides upstream of DLX1's final exon [28], we hypothesized that DLX1 undergoes NMD.

To select for our proteins in a western blot, we transfected GN11 cells with expression vectors for either Dlx1 with a 3xHA tag in the N terminus of the protein (DLX1-HA) or Dlx1(c.ins381A) with a 3x HA tag in the N terminus of the protein (DLX1(c.ins381A)-HA). We then used anti-HA antibodies to image the DLX1-HA or DLX1(c.ins381A)-HA proteins from whole-cell lysate samples. Expected protein sizes of DLX1-HA of DLX1(c.ins381A)-HA are 32.01 kDa and 16.87 kDa, respectively [34]. We then used the same membrane to image βActin as a loading control with anti-βActin antibodies.

We found that the truncated protein is translated when an expression vector for *Dlx1(c.ins381A)* with an HA tag is transfected into the cell, as evidenced by the band visible in

its corresponding lane (Figure 4). We show that the protein translated from *Dlx1(c.ins381A)*-HA is truncated by the relative sizes of the tagged proteins in the western blot, as the mutant variant is approximately 15 kDa smaller than the WT protein. Additionally, the lower intensity of the band shows that there is less of that protein present in whole cell lysate relative to the protein present in the cells transfected with an expression vector for *Dlx1* with an HA tag. These data suggest that there are pathways that are actively reducing the amount of mutant protein in the cell. This could be occurring at the level of transcription, translation, or post-translation, but this study does not identify how the cell reduces the level of protein. To further investigate our hypothesis that the truncated protein undergoes NMD, we will pursue experiments that test for specific surveillance pathways.

Because this experiment shows that there is truncated protein in the cell, our following studies are aimed at characterizing the functional capacity of the mutant protein encoded by *Dlx1(c.ins381A)*. Figure 4 was conducted using whole cell lysate, so the next step was to assess where the mutant protein is in the cell.

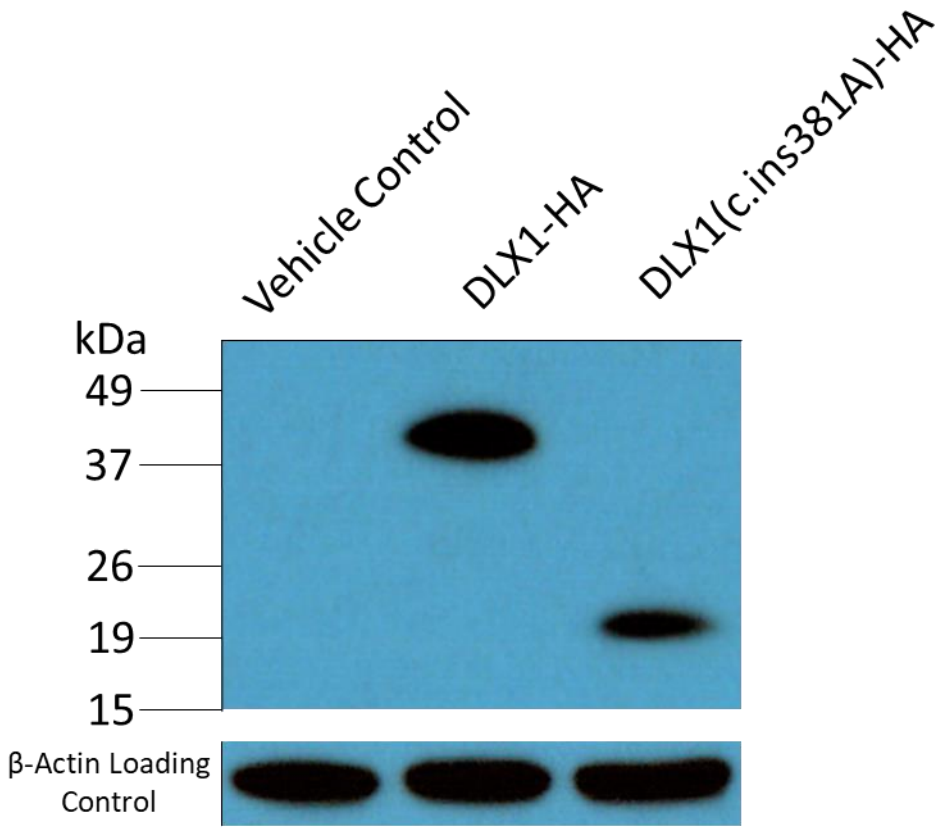

Figure 4. DLX1(c.ins381A) produces a truncated protein. GN11 cells were transfected with an expression plasmid for either DLX1 with an HA epitope or DLX1(c.ins381A) with an HA epitope. Anti-HA antibody western blot. Normalized to βActin loading control, there is less DLX1(c.ins381A) protein in whole-cell lysate.

DLX1(c.ins381A) protein partially translocates to the nucleus

DLX1 is a transcription factor, which needs to translocate to the nucleus before it regulates transcription. To assess the viability of DLX1(c.ins381A)'s transcription factor functionality, we first sought to show that the protein is in the expected location in the cell for it to be functional. To select for the WT and mutant proteins, we transfected GN11 cells with expression plasmids for either *Dlx1-HA* or *Dlx1(c.ins381A)-HA*, followed by immunocytochemistry utilizing antibodies against the HA epitope. To label the protein's location relative to the nucleus, we stained nuclei using DAPI.

Figure 5 shows that DLX1-HA does translocate to the nucleus, as expected of a transcription factor, but also that low levels DLX1(c.ins381A) translocates to the nucleus too. While the majority of the DLX1(c.ins381A)-HA protein was found in the cytoplasm, some of mutant protein is in a location where it may be able to perform its function. Figure 5 also demonstrates the previous finding in Figure 4 that there is less of the truncated protein in the cell than its wildtype counterpart. The results of this experiment suggest that the truncated protein encoded by *Dlx1(c.ins381A)* may have activity in the nucleus. The following experiments aim to evaluate the functionality of the truncated protein encoded by *Dlx1(c.ins381A)*.

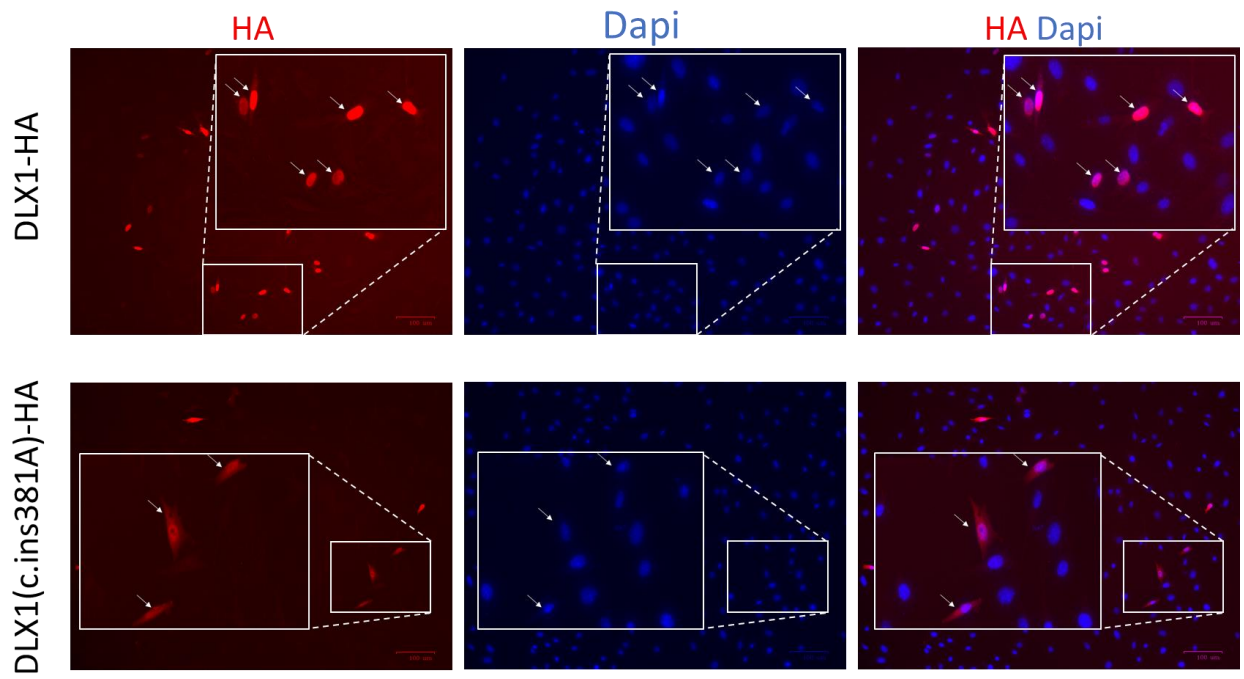

Figure 5. DLX1(c.ins381A) partially translocates to the nucleus. GN11 cells were transfected either DLX1 with HA epitope or DLX1(c.ins381A) with HA epitope expression plasmid. Immunocytochemistry was performed using anti-HA antibodies. Nuclei were labelled with DAPI. While DLX1 completely translocates to the nucleus, most of DLX1(c.ins381A)-HA protein stays in the cytoplasm and some partially translocates to the nucleus.

Dlx1(c.ins381A) Does Not Activate Homeodomain Binding-Driven Luciferase Activity

The patient that underwent WES was found to be a heterozygote with the genotype *Dlx1/Dlx1(c.ins381A)* and had abnormal puberty and a frontal lobe cognitive disorder while her WT homozygote (*Dlx1/Dlx1)* family members did not. There are two dominant mechanisms that could explain her phenotype: Haploinsufficiency (HI) or Dominant Negative (DN). A dominant negative mutation generates a mutant protein that, when overexpressed, interferes with the activity of the wildtype protein [35]. In a DN mechanism, increasing overexpression of the mutant protein would result in increasing disease phenotypes [35]. On the other hand, haploinsufficiency is characterized by a nonfunctional mutant protein and the reduced wildtype gene product is insufficient for a wildtype phenotype [35]. In contrast to DN mutations, increasing overexpression of the mutant gene will not confer a change in severity of the mutant phenotype, as increasing the amount of non-functional protein will not interfere with the function of the remaining wildtype protein.

Previous studies have established that a function of *Dlx1* is binding to homeodomain binding sites, or ATTA sites, to regulate transcription [27]. The goal of the current study is to understand the dominance mechanism to better characterize the functional effects of *Dlx1(c.ins381A)* in the heterozygous mutant. To measure the function of the wildtype DLX1 protein in conjunction with the mutant DLX1(c.ins381A), we transfected GN11, GT1-7, and NIH3T3 cells with expression vectors for *Dlx1, Dlx1(c.ins381A),* or both and co-transfected with a homeodomain binding-driven luciferase reporter.

This luciferase assay experiment is designed to (1) recapitulate the established phenotype of wildtype *Dlx1* binds to ATTA sites, now within the context of luciferase reporter expression,

(2) to characterize the functional phenotype of *Dlx1(c.ins381A)*, and (3) to suggest which dominance mechanism of *Dlx1(c.ins381A)* demonstrates.

Figure 6 shows that *Dlx1* activates ATTA site binding-driven luciferase expression. This increase in ATTA-luciferase expression is considered the phenotype of wildtype *Dlx1*. Next, we observed that *Dlx1(c.ins381A)* did not activate ATTA site binding-driven luciferase activity. When cells were transfected with both expression plasmids for *Dlx1* and *Dlx1(c.ins381A)*, luciferase activation was increased to a similar level the wildtype *Dlx1* phenotype, with no significant difference between the two conditions. These data suggest that the dominance mechanism of *Dlx1(c.ins381A)* is not dominant negative, as the presence of the mutant protein did not interfere with the phenotype of the wildtype *Dlx1*. Instead *Dlx1(c.ins381A)* could demonstrate a haploinsufficient mechanism. The next study aims to further distinguish which dominance mechanism describes *Dlx1* and *Dlx1(c.ins381A)*.

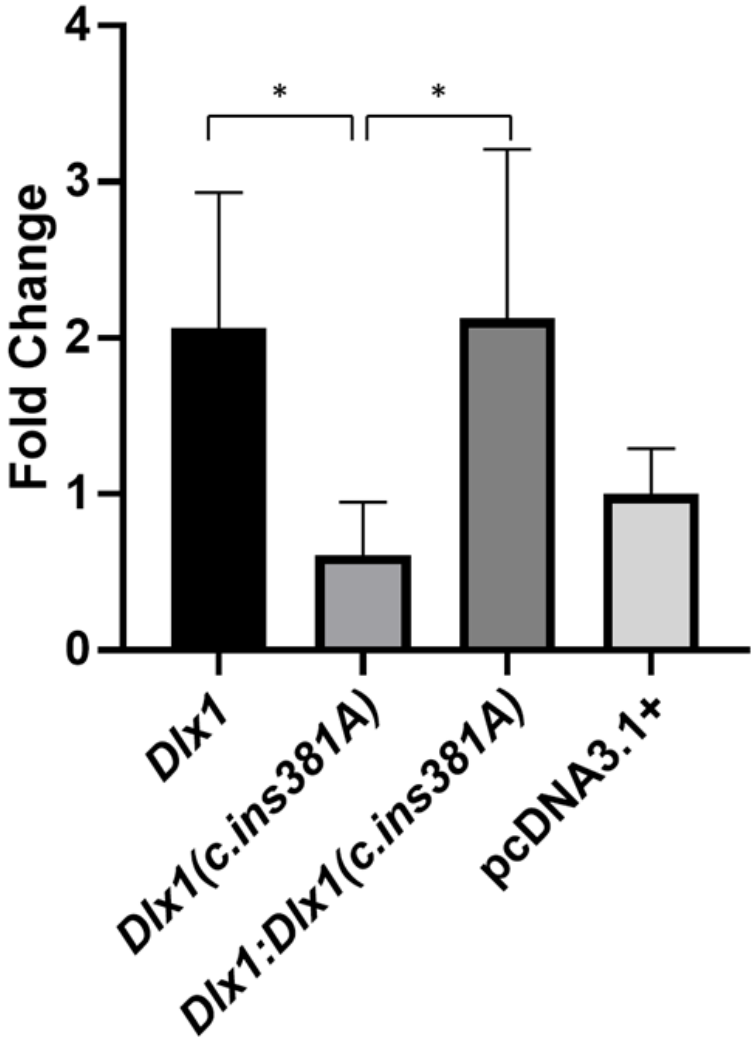

Figure 6. DLX1(c.ins381A) does not activate homeodomain binding-driven expression. GN11 cells were transfected with a reporter plasmid containing ATTA binding sites upstream of a luciferase reporter (ATTAluc). Luciferase expression could be quantified by luminance as a proxy for activation of expression by *DLX1* or *DLX1(c.ins381A)*. Cells were co-transfected with β-Galactosidase (β-Gal) expression vector for transfection efficiency control. Additionally, the cells were also co-transfected with expression plasmids for either DLX1, DLX1(c.ins381A), a mixture of DLX1 and DLX1(c.ins381A), or an empty vector pcDNA3.1+. n=5. Data were analyzed by one-way ANOVA with Tukey's post hoc analysis. *denotes $p < 0.05$.

The goal of the next experiment is to provide additional evidence to determine that the dominance mechanism of *Dlx1(c.ins381A)* is haploinsufficient and not dominant negative. A key distinguishing feature between the two is the effect of increasing overexpression of the mutant. If *Dlx1(c.ins381A)* is dominant negative, increasing the ratio of the mutant expression vector relative to the wildtype *Dlx1(c.ins381A)* expression vector should result in a decrease in ATTA-luciferase activation, as the DN mutant protein would interfere with the activity of the wildtype. If *Dlx1(c.ins381A)* is haploinsufficient, increasing the ratio of the mutant expression vector would cause no significant change to the phenotype of the wildtype.

Figure 7 shows that there is no significant decrease in ATTA-luciferase activation when we increased the overexpression of *Dlx1(c.ins381A)*. This result suggests that *Dlx1(c.ins381A)* is not dominant negative and is haploinsufficient in the heterozygote. These data indicate that *Dlx1(c.ins381A)* lacks in functionality, and thus IGD results in from insufficient transcription factor activity from the remaining *Dlx1* in the heterozygote. The next experiment aims to characterize the effect of *Dlx1(c.ins381A)* with relevance to the classical HPG axis mechanism to better understand how the mutant plays a role in propagating the IGD phenotype.

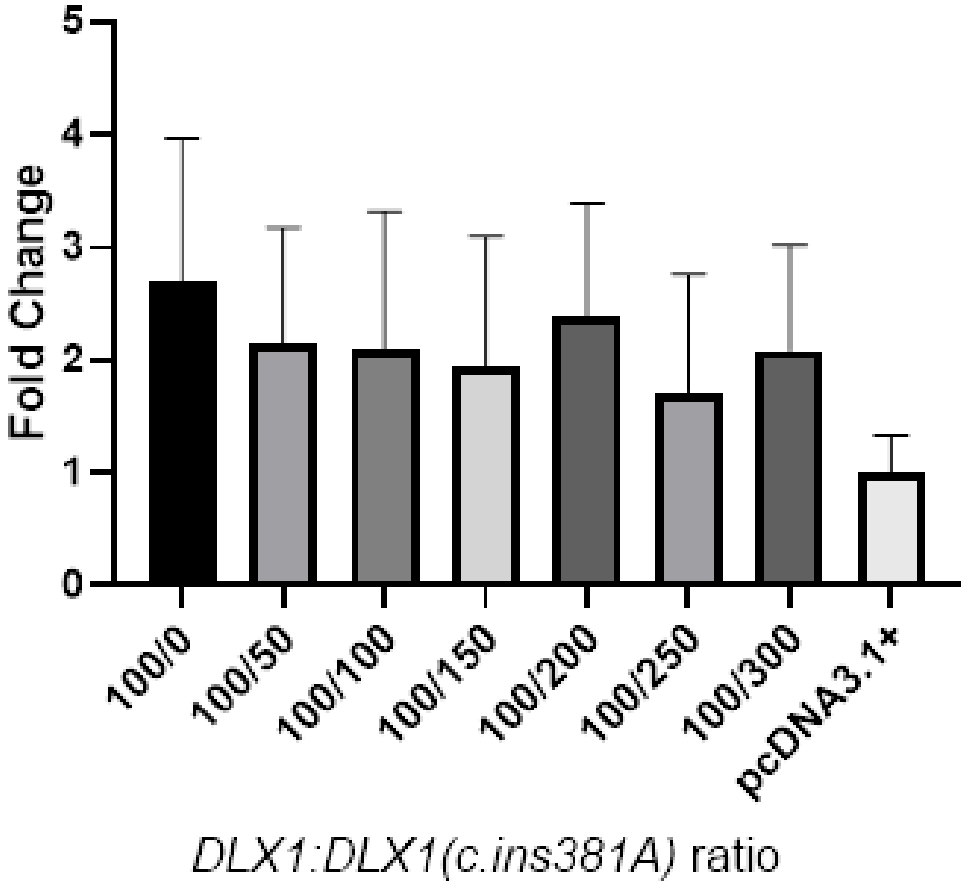

Figure 7. DLX1(c.ins381A) does not interfere with DLX1 activation of luciferase. Fibroblast (NIH3T3) cells were transfected with 100 ng of DLX1 expression plasmid and co-transfected with, ATTA-binding driven luciferase reporter plasmid, β-Galactosidase (β-Gal) expression vector and increasing amounts of DLX1(c.ins381A) expression plasmid. Fold change was calculated by luciferase expression normalized to β-Gal expression and again to empty vector (pcDNA3.1+). n=4. Data were analyzed by one-way ANOVA, n.s.

Dlx1(c.ins381A) does not activate GnRH Enhancer/Promoter-driven Luciferase Expression

The previous studies have shown that the *Dlx1(c.ins381A)* mutant does not activate homeodomain binding site-driven expression but only describes *Dlx1(c.ins381A) protein*'s capacity to function as a transcription factor. To more accurately assess the effect of *Dlx1(c.ins381A)* heterozygosity regarding fertility and the HPG axis, we performed another luciferase assay, but instead of using the ATTA-luciferase reporter, we used a luciferase reporter driven by the rat GnRH enhancer and promoter region, which contains multiple ATTA sites.

The results of this study reproduce the same luciferase activation pattern observed in the ATTA-luciferase study. Figure 6 shows that *Dlx1* activates GnRH enhancer/promoter-driven luciferase expression. *Dlx1(c.ins381A)* did not activate rGnRH e/p-driven luciferase activity. When cells were transfected with both expression plasmids for *Dlx1* and *Dlx1(c.ins381A)*, luciferase activation was increased to a similar level the wildtype *Dlx1* phenotype, with no notable difference between the two conditions. n=1, so statistical is not available currently. As this is an ongoing experiment, n is expected to rise and while the observed data continue to follow the observed trend. Thus, these data further support the hypothesis that *Dlx1(c.ins381A)* demonstrates a haploinsufficiency mechanism with relevance to the GnRH neuron function.

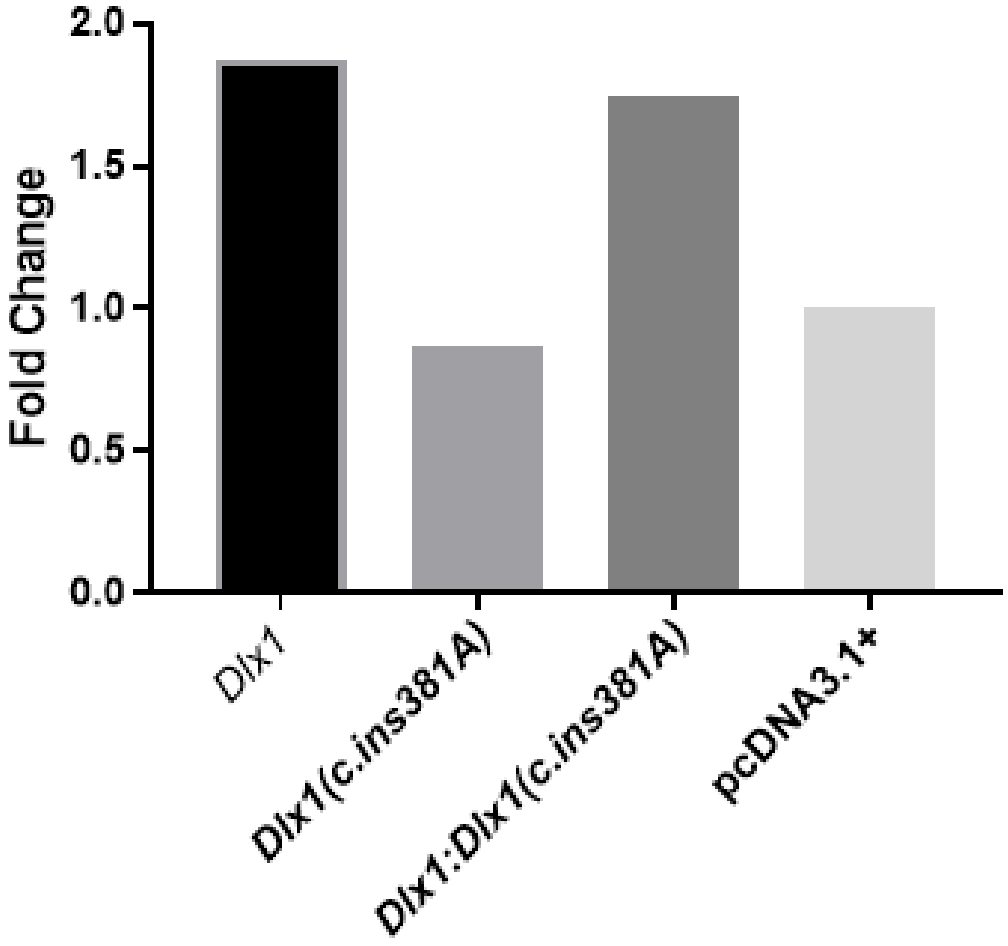

Figure 8. DLX1(c.ins381A) does not activate *GnRH* expression. Mature GnRH neurons (GT1-7) were transfected with a reporter plasmid containing the GnRH enhancer and promoter region (GnRH e/p) upstream of a luciferase reporter (GnRH-Luc). Luciferase expression was quantified by luminance as a proxy for activation of expression by *DLX1* or *DLX1(c.ins381A)*. Cells were co-transfected with β-Galactosidase (β-Gal) expression vector for transfection efficiency control. Additionally, the cells were also co-transfected with expression plasmids for either DLX1, DLX1(c.ins381A), a mixture of DLX1 and DLX1(c.ins381A), or an empty vector pcDNA3.1+. n=1. No statistical analysis possible currently.

Discussion

This study refines our current understanding of the *Dlx1/Dlx1(c.ins381A)* heterozygote. Our collaborators from the Crowley lab had revealed that a human *Dlx1/Dlx1(c.ins381A)* heterozygote had delayed puberty and low circulating sex steroids [Crowley, personal communication]. We then aimed to determine the effect of the patient mutation to better understand how the proband's genotype is connected to the observed phenotypes. In our study, we show that the *Dlx1(c.ins381A)* mutant produces a truncated protein that still partially translocates to the nucleus, where the WT protein is functionally active, but does not function properly. Our findings suggest that in the heterozygote, mutant DLX1(c.ins381A) protein will not interfere with the function of the remaining wildtype *Dlx1* activity in the context of GnRH activation, but rather lowers the overall amount of functional gene product, which we propose may result in IGD-related phenotypes.

With RT-qPCR, we screened murine-derived hypothalamic cell lines to reveal that GN11 cells, derived from developing GnRH neurons highly express *Dlx1*, which corroborates the previous data that *Dlx1* co-localizes with *GnRH* found in mouse embryos [27, 36]. Previous *in vivo* screens for *Dlx1* expression within the hypothalamus show that *Dlx1* is not expressed in ARC kisspeptin neurons [36]. Our data agrees with a previous *in vivo* study in which *Dlx1* did not co-localize with Kisspeptin transcripts in the ARC [36]. We showed that the KTaR cell line, derived from ARC Kisspeptin neurons, do not highly express *Dlx1* (Figure 3). Additionally, we show that *Dlx1* is also not highly expressed in the AVPV kisspeptin neuron cell line either (Figure 3). Interestingly, the other GnRH neuron cell line, GT1-7, did not express *Dlx1* as highly. Because GT1-7 cells are derived from mature GnRH neurons, these data suggest that a mutation in *Dlx1* may cause developmental defects in GnRH neurons. Furthermore, other studies show

that whole-body *Dlx1* KO mice have impaired craniofacial and cortical development [26,37]. The results of this experiment narrow our focus to developing GnRH neurons to more precisely describe how a loss of function mutation in *Dlx1* results in IGD phenotypes.

Figure 4 shows that there is still DLX1(c.ins381A) protein in the cell, but at lower levels, but how there is less of the mutant protein has yet to be elucidated. It could be due to less transcription, translation, or by elimination by surveillance mechanisms. It could also be that NMD is occurring but not at 100% efficiency. Nonsense Mediated Decay is not completely ruled out without an inhibition assay. The next step is to repeat the experiment, with an inhibitor of NMD, caffeine. Caffeine has been shown to reduce markers of NMD in multiple cell lines, including Human leukemia K562 and HL60 cells, Human embryonic kidney 293 cells [38, 39] and HeLa cells [39, 40]. We would transfect GN11 cells with the expression vector for DLX1(c.ins381A)-HA and treat the cells with caffeine or vehicle control, then collect protein for western blot using anti-HA antibodies. Our hypothesis that DLX1(c.ins381A) undergoes NMD would be supported if the intensity of the band for the caffeine-treated cells is darker than that of the vehicle control, as that would suggest that NMD suppression by caffeine reduced the decay of translating *Dlx1(c.ins381A)-HA* transcripts.

Because there is mutant protein in the cell, albeit less, we next inquired if the truncated DLX1(c.ins381A) localizes to where wildtype DLX1 localizes. As a transcription factor, DLX1 translocates to the nucleus, where it can then regulate transcription [41]. Our results are consistent with the results of Chiba and colleagues' previous study, but with HA-tagged DLX1 proteins (Figure 5). Our results show that HA-tagged DLX1 protein (DLX1-HA) translocates to the nucleus and that the 3xHA tag in the N-terminus does not interfere with the proteins ability to translocate to the nucleus. Chiba et al. had established that DLX1's homeodomain region is

essential to the protein's ability to translocate to the nucleus, as complete removal of the homeodomain resulted in complete cytoplasmic localization [40]. *In silico* analysis of *Dlx1(c.ins381A)* shows that DLX1(c.ins381A) protein causes a nonsense mutation resulting in a PTC, but before the PTC, the protein contains only the first 5 amino acids of the homeodomain followed by 2 incorrect amino acids before the stop codon. Because the mutant protein was missing a significant portion of the homeodomain region, we hypothesized that it would not translocate to the nucleus.

Figure 5 revealed that low amounts of DLX1(c.ins381A) protein were detected in the nucleus, while most of the detected protein was found in the cytoplasm. Unlike the previous study conducted by Chiba and colleagues, DLX1(c.ins381A) had a minimal portion of the homeodomain region, as opposed to their complete removal of it. Our results suggest that the minimal amount of homeodomain region that the mutant protein has is sufficient to allow some of the protein to translocate to the nucleus, where transcription factors, including DLX1, regulate transcription. This led us to investigate the functional capacity of *Dlx1(c.ins381A).*

Because *Dlx1* is known to be a transcription factor, we first sought to evaluate its ability to function as a transcriptional activator. Since homeodomain transcription factors regulate transcription by binding to (ATTA) sites [25], we chose to use a luciferase reporter driven by ATTA site binding. To assess *Dlx1(c.ins381A)'s* functionality, we first recapitulated the previous finding that *Dlx1* overexpression upregulates expression of the luciferase reporter when it is driven by ATTA-binding activity. Using this model, we could measure if the *Dlx1(c.ins381A)* is able to upregulate ATTA-luciferase reporter activity like its wildtype counterpart. Figure 6 shows that *Dlx1* does not activate ATTA-luciferase expression, suggesting that *Dlx1(c.ins381A)* is likely unable to function as a transcription factor.

A caveat to our ATTA-luciferase experiment is that it only shows that *Dlx1(c.ins381A)* cannot activate homeodomain binding-driven luciferase activity and that doesn't convey the protein's ability to bind directly to ATTA sites. To further assess *Dlx1(c.ins381A)*'s function within the context of the homeodomain, our next experiment will investigate DLX1(c.ins381A)'s ability to bind to ATTA sites using an Electrophoretic Mobility Shift Assay (EMSA). With an EMSA, we will directly assay for the protein-DNA interaction between DLX1 or DLX1(c.ins381A) and the ATTA sites in the GnRH enhancer region in GN11 cells. As previously demonstrated, DLX1 binds to radiolabeled DNA probe corresponding to the *GnRH* homeodomain binding site (−1642/−1623), which contains two ATTA sites [27].

To study DLX1 and DLX1(c.ins381A), we will collect nuclear extracts from NIH3T3 cells transfected with expression plasmids for DLX1-HA or DLX(c.ins381A)-HA then incubate the samples with the probe corresponding to the *GnRH* homeodomain binding site. To identify the protein-DNA complexes that contain DLX1-HA or DLX1(c.ins381A), we can supershift by including another condition in which anti-HA antibodies are added to the incubation reaction. After incubating the transfected cells' nuclear extract with the probes containing ATTA sites, we can compare the banding patterns between the samples incubated with and without anti-HA antibodies. Bands that supershift with the antibody would signify a supershift complex containing the HA-tagged protein, as validated by anti-HA antibody binding. We expect to observe a supershifted complex in the *Dlx1-HA* +antibody condition, recapitulating the previous finding that DLX1 binds to ATTA sites. Thus, the lack of a difference in banding pattern between the *Dlx1(c.ins381A)-HA* transfected nuclear extracts with and without the antibody would indicate that DLX1(c.ins381A) fails to bind to ATTA sites or complexes that bind to ATTA sites.

The previous study was performed on nuclear extracts from NLT cells, which are derived from the same tumor in developing GnRH neurons as GN11 cells [42]. However, endogenous DLX1 may compete with the probe-protein binding, which is a concern, as Figure 3 has shown that *Dlx1* RNA is highly enriched in GN11 cells. Thus, we can reduce the risk of endogenous DLX1 binding competition by first performing this assay in NIH3T3 cells, which express *Dlx1* at lower levels. A follow up study to this experiment then is to repeat the EMSA using GN11 cells, as they model neurons that are relevant to *Dlx1*'s role in the reproductive axis.

Our luciferase experimental design also allowed us to study the functional consequences of co-expressing *Dlx1* and *Dlx1(c.ins381A)*, which models a heterozygous individual, expressing one of each allele. To reiterate, the proband of the WES study was found to be heterozygous for *Dlx1/Dlx1(c.ins381A)*. To characterize the interaction between the alleles of the heterozygote, we aimed to determine if the dominance mechanism of the mutation was either dominant negative or haploinsufficient. Our data in Figures 6, 7, and 8 suggest haploinsufficiency, as the phenotype associated with the homozygous WT allele is observed in the heterozygous condition. In a titration study increasing the ratio of *Dlx1(c.ins381A)* to *Dlx1* co-transfection, we observed no significant change in ATTA-luciferase activity, suggesting that increasing amounts of mutant protein does not affect the function of the wildtype, therefore our results follow the classical definition of a haploinsuffient mutation. A follow up experiment to further characterize *Dlx1(c.ins381A)* and *Dlx1(c.ins381A)* heterozygosity with regards to *Dlx1*'s function of regulating GnRH expression is to repeat the titration luciferase assay, but with GnRH e/p-luciferase.

It should be mentioned that our experiments do not completely rule out the dominant negative mechanism. Another proposed possibility to explain the dominance mechanism is a

subtype of DN mutations in which the mutant protein exhibits an off-target effect that causes a disease-related phenotype. As previously established by Givens et al., *Dlx5* is known to activate GnRH e/p-luciferase activity. Additionally, knockout of both *Dlx1* and *Dlx2* results in decreased expression of *Dlx5* in mouse embryos [43]. For this reason, we can pose the alternative hypothesis that *DLX1(c.ins381A)* interrupts *Dlx5's* expression in a dominant-negative fashion, interrupting *Dlx5* expression and thus its ability to activate GnRH expression, propagating IGD-related phenotypes. To test this hypothesis, we can design a luciferase reporter plasmid that is driven by the *Dlx5* promoter, creating a proxy to measure changes in *Dlx5* expression when either *Dlx1, Dlx1(c.ins381A),* or both expression vectors are transfected into the cells. If this mechanism is dominant negative, we expect to see that *Dlx1* does increase *Dlx5-luciferase* activity, *Dlx1(c.ins381A)* does not do so, and that when cells are co-transfected with both expression plasmids, the increase in *Dlx5*-luciferase would be attenuated. Furthermore, if the dominance model is DN, a titration of increasing the ratio of *Dlx1(c.ins381A)* expression plasmid to *Dlx1* expression plasmid, would reveal that increasing overexpression of *Dlx1(c.ins381A)* would result in more severe decreases in *Dlx5-luciferase* activity. To support our hypothesis that *Dlx1(c.ins381A)* is haploinsufficient, we expect to observe no significant change in *Dlx5-luciferase* activity with increasing overexpression of *Dlx1(c.ins381A)*.

The next step in our studies is directly assessing the functionality of the protein encoded by *Dlx1(c.ins381A)*. Previous studies have established that DLX1 binds to another protein, SMAD4 and that the homeodomain is essential to this protein-protein interaction [41]. In their study, deletion of the homeodomain from DLX1 prevented DLX1-SMAD4 binding [41].

Dlx1(c.ins381A) produces a truncated protein that lacks a significant amount of the homeodomain due to the PTC. To determine if DLX1(c.ins381A) is functional in the context of

protein-protein interaction, we can perform an experiment that combines immunoprecipitation (IP) with western blot. We can transfect expression plasmids for *Dlx1* and *Dlx1(c.ins381A)* with HA tags into cells that express SMAD4, then collect the protein of the cells and select for DLX1-HA and DLX1(c.ins381A)-HA in a pull-down using IP beads with anti-HA antibodies. After that, we can screen the pulled-down proteins for SMAD4 in a western blot using anti-SMAD4 antibodies. The appearance of bands in that experiment in the *Dlx1-HA* expression plasmid-transfected condition would recapitulate established findings by other studies that DLX1 binds to SMAD4. The western blot will reveal if the protein encoded by *Dlx1(c.ins381A)* can still bind to SMAD4.

Previously, *in vivo* studies of *Dlx1* in the hypothalamus were in homozygous *Dlx1/2* knockout (KO) mice [27]. That is, the knockout targeted both *Dlx1 and Dlx2*. To more accurately model the DLX1(c.ins381A) proband, we can generate $Dlx1^{+/-}$ heterozygous (HET) mice. The previous study has established that co-expression *Dlx1* and *Dlx2* is essential to GnRH neuron migration and fertility, but the effect of only having one functional copy of *Dlx1* has not been studied *in vivo*. Combined with our findings that *Dlx1(c.ins381A)* is haploinsufficient (Figures 6-8), we can deepen our understanding of how the HET genotype results in IGD phenotypes. We can perform pubertal onset, fertility, and hormone assays in *DLX1* HET mice and compare HET phenotypes to those of the WT and *Dlx1* KO mice, which would also be the first study to characterize the reproductive phenotypes of the *Dlx1* KO mice. Furthermore, we would determine if the heterozygous mice would exhibit phenotypes comparable to the molecular phenotypes observed in the current study. Relative to the homozygote *DLX1* KO mice, which die before 3 weeks of age, *DLX1* HET mice are expected to display a milder phenotype. Additionally, we can collect *Dlx1* HET embryos at different embryonic ages to quantify GnRH

neurons and determine the migratory pathway of developing GnRH neurons in *Dlx1* HET embryos via *in situ* hybridization (ISH) using probes against *GnRH* transcripts. These experiments will advance our knowledge of the role of *Dlx1* in reproduction and build on our understanding of the effects of lacking a functional copy of *Dlx1*.

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