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The effect of homeodomain genes *Six3* and *Six6* on GnRH neuronal migration and apoptosis

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

in

Biology

by

Lauren Diane Sitts

Committee in charge:

Pamela L. Mellon, Chair
Nicholas C. Spitzer, Co-Chair
James E. Cooke

2017

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The Thesis of Lauren Diane Sitts is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

Co-Chair

Chair

University of California, San Diego

2017

DEDICATION

I would like to dedicate this thesis to my Mom, Dad, and Brother. Thank you for your unconditional love, support, positivity, and guidance. Without all of you, I don't know where I would be.

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LIST OF ABBREVIATIONS

Ark	Adhesion related kinase
Dach1	Daschund 1
E	Embryonic day
EphA5	Ephrin A5
Fgfr1	Fibroblast growth factor receptor 1
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HET	Heterozygous
HPG	Hypothalamic-pituitary-gonadal
IHC	Immunohistochemistry
IHH	Idiopathic hypogonadotropic hypogonadism
KO	Knockout
LH	Luteinizing hormone
MOB	Main olfactory bulb
MOE	Main olfactory epithelium
Nelf	Nasal embryonic LHRH factor
Npn1	Neuropilin 1
Npn2	Neuropilin 2
Otx2	Orthodenticle homeobox
Prok2	Prokineticin 2
Sema3A	Semaphorin 3A
Six3	Sine oculis-related homeobox 6
Six6	Sine oculis-related homeobox 6
T	Testosterone
Vax1	Ventral anterior homeobox
WT	Wild type

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Special thanks to my other committee members, Dr. James Cooke and Dr. Nicholas Spitzer, for their interest in my project and support.

ABSTRACT OF THE THESIS

The effect of homeodomain genes *Six3* and *Six6* on GnRH neuronal migration and apoptosis

by

Lauren Diane Sitts

Master of Science in Biology

University of California, San Diego, 2017

Professor Pamela L. Mellon, Chair
Professor Nicholas C. Spitzer, Co-Chair

Infertility affects between 8-12% of couples worldwide and treatment can be very costly. One cause of infertility is Idiopathic Hypogonadotropic Hypogonadism (IHH), which can arise from the loss of gonadotropin-releasing hormone (GnRH). Many of the genes associated with IHH are currently unknown. *Six3* and *Six6* are genes associated with infertility in mouse models. Disruption of either *Six3* or *Six6* causes a decrease in the number of GnRH neurons found in the

hypothalamus. The *Six3* heterozygous mouse loses GnRH neurons to mismigration, whereas the *Six6* knockout mouse loses GnRH neurons to apoptosis. To provide further insight into the mechanisms for the loss of GnRH neurons, we studied cell migration and apoptosis factors present along the migratory pathway of GnRH neurons. Our hypothesis was that, without adequate levels of these factors, GnRH neurons may die, become lost, or halt migration. We found that haploinsufficiency of *Six3* leads to a trend towards a reduction in *Npn2* and *Nelf* mRNA expression within the hypothalamus, and both of these factors have been shown to have a significant effect on the guidance of GnRH neurons. We found that complete loss of *Six6* leads to a trend toward reduction in apoptotic factor *Dach1*, which is a potential novel regulator of GnRH neurons. Overall our findings support the idea that *Six3* and *Six6* affect the migratory environment that supports GnRH neuron migration from the olfactory placode to the hypothalamus. Additionally, our findings implicate the genes, *Npn2*, *Nelf* and *Dach1,* in the regulation of GnRH neurons during *Six3* and *Six6* disruption.

INTRODUCTION

The role of gonadotropin-releasing hormone in neuroendocrinology

The hypothalamic-pituitary-gonadal axis (HPG) is a feedback loop that is essential for the maintenance of fertility and puberty (Figure 1). At the apex of this feedback loop, gonadotropin-releasing hormone (GnRH) is secreted from GnRH neurons in the hypothalamus. GnRH is released in a pulsatile manner into the hypophyseal portal system [1]. GnRH then travels to the anterior pituitary where it will activate gonadotropes to stimulate release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [2, 3]. FSH and LH then act on the gonads to stimulate the release of testosterone, estrogen, and progesterone. These sex steroids then negatively feedback onto the hypothalamus in to inhibit further secretion of GnRH [2]. Adequate levels of sex steroids are necessary for the processes of spermatogenesis and folliculogenesis, and therefore are needed to maintain fertility. Interestingly, male mice can have an 87% reduction in GnRH neurons and still present with normal fertility, whereas female mice can only withstand a 66% reduction in GnRH neurons and still exhibit normal fertility [4].

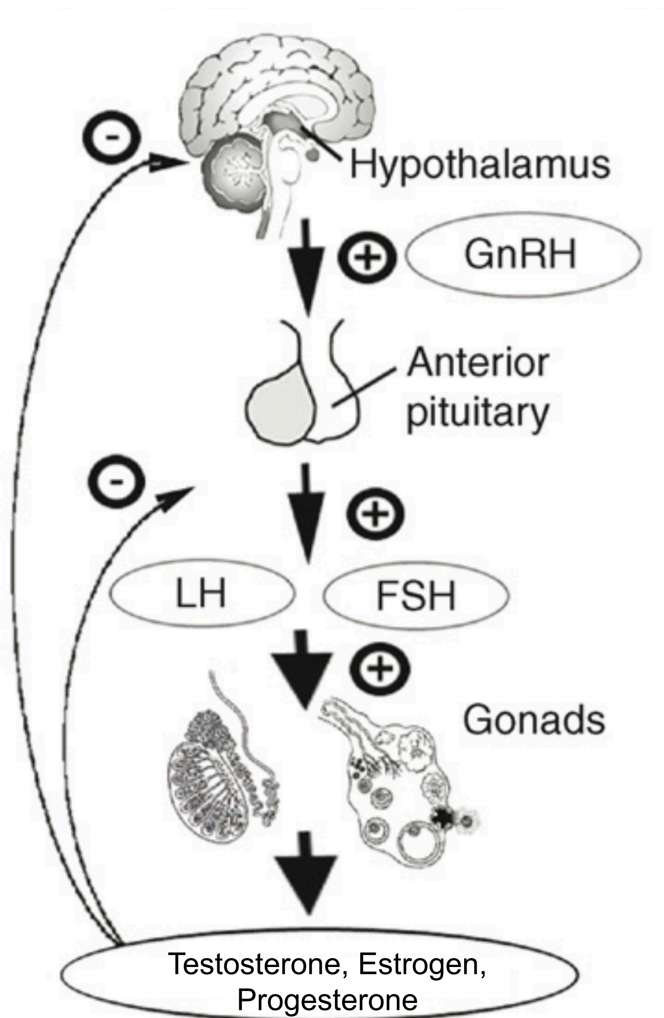

Figure 1: Hypothalamic-Pituitary-Gonadal Axis. Signaling occurs through a series of feedback loops. GnRH is secreted from the GnRH neurons of the hypothalamus and acts upon the anterior pituitary to stimulate the release of LH and FSH. These factors will then act upon the testis and ovaries to stimulate the release of hormones like testosterone, estrogen and progesterone [2, 3] (adapted from [5]).

GnRH Neuron Migration

In mammals, GnRH neurons originate within the nose in the olfactory placode. In mice, GnRH neurons emerge between embryonic day 11.5-12 (E11.5-E12) [5, 6]. GnRH neurons migrate out of the olfactory placode, through the main olfactory epithelium (MOE), across the cribriform

plate, and into the hypothalamus along the axonal projections of olfactory neurons (Figure 2). The peak of this migration is at E15.5 and is completed at E18.5. From the hypothalamus, GnRH neurons will release GnRH to drive the HPG axis [5].

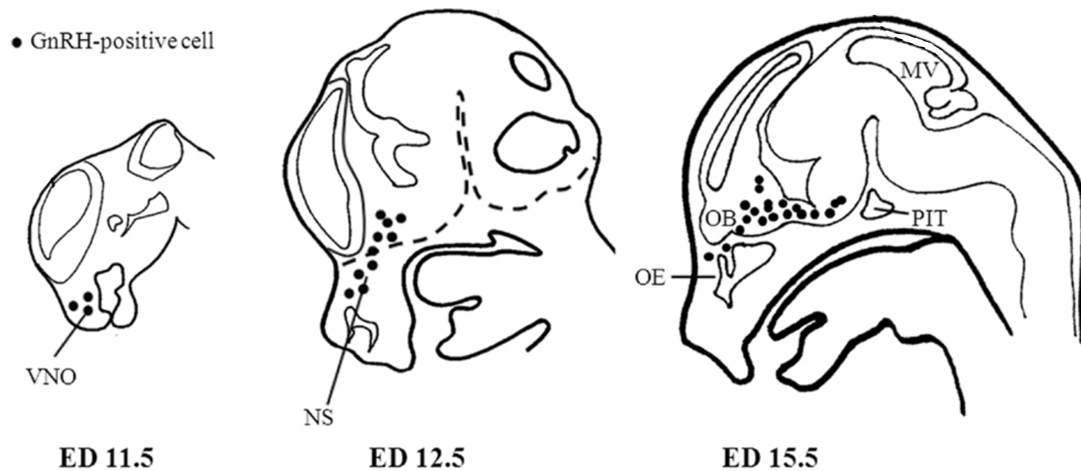

Figure 2: GnRH neuronal migration. GnRH neurons (black dots) are migrating from the olfactory placode to the hypothalamus. Intermediate points of migration are shown with the peak of migration is at E15.5. [6].

Since GnRH has a direct effect on the reproductive functions of the body, alterations in levels of GnRH can result in significant effects on fertility [7]. When a GnRH neuron does not make it to the hypothalamus, GnRH cannot be released to the hypophyseal portal system. The loss of GnRH can lead to the condition Idiopathic Hypogonadotropic Hypogonadism (IHH). The condition of IHH is rare, being present in only 0.0001% of all live births [8]. IHH is characterized by infertility and delayed or absent puberty. There is a subtype of IHH, Kallmann's syndrome, in which infertility is paired with anosmia, or the inability to detect smells. This condition represents ~60% of cases of IHH, and is believed to be linked to GnRH neuron development and migration in

the nose. Therefore, when there are developmental impairments of the olfactory system, GnRH neurons can also be lost [8].

Infertility, including in couples with IHH, affects between 8-12% of couples worldwide, with some countries having infertility rates as high as 30% [9]. Humans are diagnosed as infertile when they have been trying unsuccessfully to conceive a child for at least one year. The percentage of people who are plagued by infertility is still rising, yet many of the genetic causes of infertility are not understood. For example, the majority of cases of IHH and Kallmann's syndrome are of an unknown genetic origin, with many of the cases being causes by defects in more than one gene [10]. As a result, this presents the need to discover new genes that may be associated with these conditions of infertility. Two genes that have been demonstrated to play a role in GnRH migration and whose role is only beginning to be appreciated but have not been well-studied in reproduction are *Six3* and *Six6*.

Six3 and Six6

The sine oculis-related homeobox *(Six)* family of genes is found in many species and is composed of three subgroups *Six1/2*, *Six3/6*, and *Six4/5*, all of which are critical during embryogenesis. This family of genes is important during embryogenesis for a variety of structures. It is also important to note that *Six1*, *Six2*, *Six4*, and *Six5* all have a very broad expression during embryogenesis [11]. In contrast, both *Six3* and *Six6* are localized to the brain and eye during embryogenesis, where they are critical regulators of eye and forebrain development. In zebrafish mutants that have a headless phenotype, injections of Six3 are capable of rescuing this phenotype because of its ability to repress Wnt1, which is a factor that suppresses development of the forebrain [12]. The *Six3/6* subgroup has also been to be implicated in GnRH neuronal migration and survival [11]. Six6 is highly increased during GnRH neuronal maturation and overexpression results in

GnRH transcription within neuronal cells [13]. Furthermore, the transcription of GnRH is meditated through the binding of Six6 to the sites within the GnRH proximal promoter [13].

Both *Six3* and *Six6* are homeodomain genes and contain a *Six* domain and a homeodomain, which are needed to bind DNA and specific cofactors [14]. The homeodomain is unique in that it does not contain two amino acid sequences that are essential for binding the TAAT sequence [11]. At the beginning of embryogenesis, *Six3* and *Six6* have overlapping expression patterns but as development progresses, at E17.5 their expression becomes more differentiated [14] (Figure 3). *Six3* expression is also found in the olfactory bulb, hippocampus, cortex, and several other areas of the brain, whereas *Six6* had lower expression levels in these regions [14]. It is also important to note that the expression of the *Six3* and *Six6* genes have a balance in their expression profiles and it is thought that the genes may be compensating for one another.

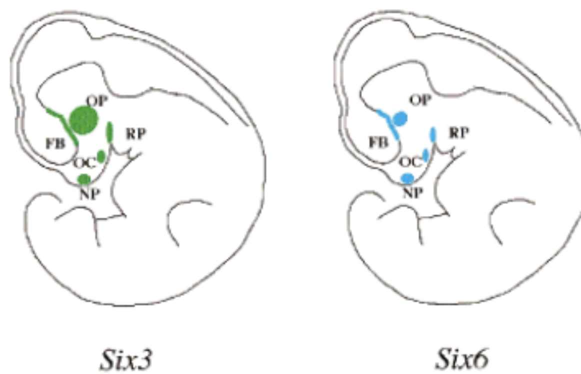

Figure 3: Homology amongst expression of homeodomain genes *Six3* and *Six6* during embryogenesis. Image is indicating the overlapping expression patterns seen in early embryogenesis. Expression is seen in the areas where GnRH neurons are born and where they will migrate to. NP, nasal placode; FB, forebrain; OP, optic vesicle; OC, optic chiasm; RP, Rathke's pouch [11].

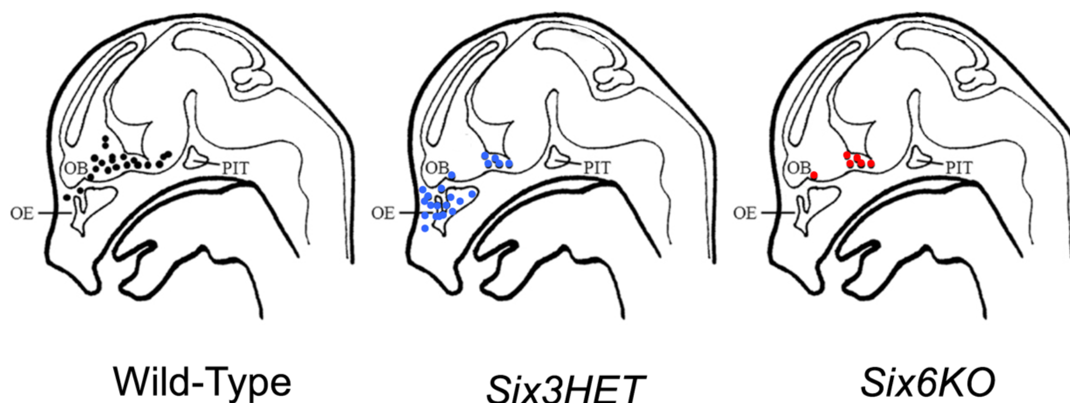

Figure 4: GnRH Neuron loss in *Six3*HET and *Six6*KO mouse models at E15.5. Wild-Type (WT) mice (left) with normal GnRH neuron migration. SixHET mouse (middle), indicates GnRH neurons that are halted in migration. *Six6*KO model showing significant loss of GnRH neurons, but they were able to migrate out of the nose (right) (adapted from ([6]).

Six3

Six3 is found on chromosome 17 and is essential in forebrain and eye development [11]. *Six3* has been linked to holopresencephaly, which is a condition characterized by malformation of the brain and craniofacial abnormalities [11]. (Figure 5). The major brain/face structural abnormalities result in the Six 3 knock-outs to be post-natal lethal (Figure 5) [12].

Six 3 knock-outs are post-natal lethal and have major brain/face structural abnormalities (Figure 5); for this reason, the role of Six3 can be examined in *Six3* heterozygous (*Six3*HET) mice instead. These mice are also anosmic, have a 45% decrease in GnRH neurons in the hypothalamus, and have a decrease in fertility. The GnRH neurons are halted in migration within the olfactory placode in the *Six3*HET mouse model (Figure 4). In males, the decrease in fertility is not known to be due to irregular levels of hormones, but rather is behavioral. Many animals are driven by pheromones and scents, especially when it comes to mating. Six3HET mice did not attempt to mount the female, which may be due to their inability to detect pheromones. This infertility in

males likely arises from anosmia, rather than a disruption in the HPG axis. As a result, they rarely produce litters. The Six3HET females are significantly less fertile than their WT counterparts, due in part to an irregular estrous cycle (Pandolfi and Mellon, unpublished data).

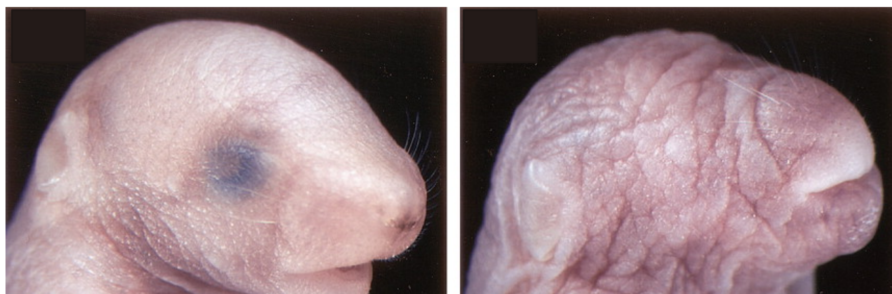

Figure 5: Image of *Six3*KO mice. Newborn wild type mouse (left) and Six3KO mouse (right). Postnatal lethal one day after birth (Image captured at P1). This is the result of lacking formation of eye, forebrain, and other craniofacial abnormalities [12].

Six6

Six6 is found on chromosome 14 and is associated with anopthalmia [11]. Anopthalmia is a condition that is associated with loss of the optic nerve and abnormalities within the pituitary. *Six6*KO mice have an 89% decrease in GnRH neurons. [13]. The GnRH neurons are able to migrate out of the nose, but at some point in development they are lost due to apoptosis (Figure 4). Both sexes of the *Six6*KO mice are infertile likely due to the significant reduction in GnRH neurons. The female *Six6*KO mice have smaller and fewer corpora lutea, and irregular estrous cycling. The male *Six6*KO mice have lower levels of FSH, LH and T [13].

We believe that there is an alteration in the factors along the migratory pathway of GnRH neurons resulting in the loss of GnRH neurons in the *Six3* and *Six6* mouse models. Therefore, we used qRT-PCR to determine the mRNA expression levels of migratory/apoptotic factors within adult hypothalamic micropunches. Factors that were seen to have a difference in gene expression,

were then examined using immunohistochemistry (IHC) in E14.5 mice to determine the presence of the proteins along the migratory pathway.

MATERIALS AND METHODS

Mouse Lines

All mice were housed at UCSD according to the Institutional Animal Care and Use Committee guidelines. The mice were housed in groups on a 12-hour light/dark cycle, while being fed chow and water *ad libitum*. *Six3* mice were graciously provided by Dr. Oliver Guillermo's lab and *Six6* mice were graciously provided by Dr. Geof Rosenfeld's Lab. Genotyping was performed with the 5'-3' primers:

*Six6*F:AGACAGACTGCATTCCCAGC *Six6*R1:AGCCTGAAGAACGAGATCAGC *Six6*R2:AGACTCACTGCTTCAAGGAGC

*Six3*F:CCCCTAGCCTAACCCAAACATTCG *Six3*R1:TTCCCCTCTTTGACTCCTATGGACG *Six3*R2:CGGCCCATGTACAACGCGTATT

Timed Mating

Mice were housed in trios with two females and one male per mating cage. Females were checked every morning for a copulatory plug and placed in individual cages when after a plug was observed.

Embryo Harvesting

Embryos were collected from the timed matings. Embryos used for immunohistochemistry (IHC) were placed in a fixative solution of 60% ethanol, 30% formaldehyde and 10% glacial acetic acid.

Brain Collection

For the cryostat, brains were immediately placed on dry ice after collection. They were then embedded in Tissue-Tek O.C.T. Compound (Sakura) and sagittal sections were placed on SuperFrost Plus slides (Thermo Fisher Scientific). 10 µm sagittal sections were taken and the hypothalamus was micropunched with a 3 mm punch. Only a half crescent punch was taken from the ventral side of the brain. Punched tissue was placed directly into RNAlater Stabilization Solution (Thermo Fisher Scientific).

Quantitative RT-PCR

Total mRNA was isolated using RNAqueous-Micro Kit (Invitrogen). Purified RNA was then synthesized into cDNA using iScript cDNA Synthesis Kit (BioRad). cDNA was made to a concentration of 50 ng/µl. When testing for the expression of the different genes (See table1), cDNA was diluted 1:20 for all factors, except *prok2*, which had a cDNA dilution of 1:10. Quantitave RT-PCR (qRT-PCR) was performed on CFX Connect Real-Time System (Bio-Rad Laboratories). All primers were run on the protocol: one cycle of 95°C for 3 minutes, after which there were 39 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Threshold data was collected at the 60°C annealing phase of each repeated cycle. Data were analyzed using the ddCt method [13]. Values were first normalized to the housekeeping gene *H2afz* and then they were normalized to the wild type. *Fgfr1* primer was purchased from GeneCopoeia (Catalogue #MQP029161). *Dach1*, *Nelf*, and *Npn2* primers were obtained through Mouse Primer Depot (https://mouseprimerdepot.nci.nih.gov/). Primers for *EphA5*, *Otx2*, *Prok2* and *p27kip1* were

obtained through Primbank (https://pga.mgh.harvard.edu/primerbank/). Primers for *Sema3A* [15], *Ark* [16], *H2afz* [17], and *Vax1* [17] have been previously published.

Table 1: Primers of factors used for qRT-PCR. Annealing temp: 60°C

Factor	Forward (5'-3')	Reverse (5'-3')
Nelf	AGCGTTGATGGAGTGTCTGAT	GTGTTTGCGGAAGTTCGATA
Npn2	TGTTCGAGGGAGTGATAGGG	ATCCTCACCTGCAAAAGCTG
EphA5	GAGCCAGATCGCCCCAATG	TCGGCTGTAATACTGGTCTCTTT
Sema3A	TGTTCATCGGAACAGATGTTGGAA	GATGATTCTCTCTTCAAGGCTGGG
Prok2	GCCCCGCTACTGCTACTTC	CCCCGTGCAGACACTAACTTT
Vax1	CCGGATCCTAGTCCGAGATGCC	TCTCCCGGCCCACCACGTAT
Otx2	GCAGAGGTCCTATCCCATGA	CTGGGTGGAAAGAGAAGCTG
Dach1	TCCAGGAAGGACTTCGAGAC	GATTCCAGGAGACATGAGGC
Ark	TTAAATGCCCAAGAATACA	GAGGCACCAGAGTCCAC
P27kip1	TCAAACGTGAGAGTGTCTAACG	CCGGGCCGAAGAGATTTCTG
H2afz	TCACCGCAGAGGTACTTGAG	GATGTGTGGGATGACACCA

Immunohistochemistry

The protocol for IHC has been previously described in (Hoffmann et al., 2014). Antigen retrieval was performed using 10 mM sodium citrate. Primary antibody concentrations used were Npn2 1:300 (Cell Signaling Technology, #3366), Npn1 1:300 (Abcam, ab81321), Nelf 1:1000 (Sigma Aldrich, SAB3501030). Secondary antibody concentrations used were goat anti-rabbit 1:300.

Statistical Analysis

All analysis was performed using Prism (Graphpad, San Diego CA). Values were first normalized to the housekeeping gene and then they were normalized to the wild type. A student's

t-test was then performed to determine statistical significance, with significance indicated by $p < 0.05$ and trending toward significance with $p < 0.1$.

CHAPTER 1. Factors associated with the mismigration of GnRH neurons in *Six3*HET mice

1.1 Introduction

As previously stated, in the *Six3* mouse model, the majority of GnRH neurons do not make it to the hypothalamus because they are halted in migration and remain within the nose. It has also been observed that an alteration in the presence of guidance factors along the migratory route can alter the migration of GnRH neurons [10, 18-21]. Thus, we speculated that there may be an alteration in various axonal guidance factors. To elucidate the mechanism by which GnRH neurons are halted or move off of their migratory path in the *Six3HET* mouse model, we studied a variety of factors that have been known to be associated with GnRH neuronal migration. Some of these factors are specific to guiding the neurons out of the nose and others are implicated in aiding the GnRH neurons while migrating through the brain. While there are a multitude of factors that could be implicated in the phenotype observed in the *Six3*HET mice, we decided to focus on the genes most likely to be altering migration in this mouse model.

Factors associated with neuronal migration

Along the journey to the hypothalamus, GnRH neurons encounter many different molecular environments. Therefore, it is essential that factors are present along this pathway to aid the neurons in making it to the correct destination and survive along the pathway. Some factors will be essential in the development of olfactory projections, to indirectly affect the migration of the GnRH neurons [19], while chemoattractants, chemorepellents, peptides, neurotransmitters, and transcription factors may directly affect the migration of the GnRH neurons [19].

It is first essential that GnRH neurons are able to leave the olfactory placode and travel along the vomeronasal nerve through the cribriform plate. NELF (nasal embryonic LHRH factor) is necessary for GnRH movement along the vomeronasal nerve as experiments that have blocked the expression of *NELF* have also been seen to have an effect on GnRH neuronal migration [5].

Ephrin receptor tyrosine kinases have been implicated in having attractive and repulsive effects that can contribute to axonal guidance [22]. In the mouse brain, *EphA5* seems to have a profound effect on the migration of GnRH neurons from the olfactory placode to the hypothalamus. Overexpression of *EphA5* at E11 slowed GnRH neuron migration and promoted clustering of the neurons amongst the olfactory axons, which inhibited the GnRH neurons from migrating out of the nose. This caused a reduction in GnRH neurons and led to infertility.

Npn2 (*Neuropilin-2*) is involved in axonal guidance and is also a co-receptor for class 3 semaphorins [5]. Research has shown that *Npn2* KO mice have an accumulation of GnRH neurons within the nasal compartment, which is a characteristic that is consistent with the *Six3*HET mice [5]. Furthermore, it has been seen that *Npn2* may interact with ligands to cause proper fasciculation of the vomeronasal nerve [5], which is the main pathway that GnRH neurons use to travel out of the nose toward the olfactory bulb.

Semaphorins act as pathfinding factors to guide neurons [23]. *Semaphorin 3A* (*Sema3A*) is a protein that is secreted from the membrane and works with *Npn*-1 axons to have repulsive properties [21]. It has also been found that a deletion of *Sema3A* leads to phenotypes similar to that seen in Kallmann's syndrome.

Fibroblast growth factor 1 (Fgfr1) mutations have been seen in some individuals that have been diagnosed with Kallman's syndrome [24]. *Fgfr1* has also been implicated with the normal development of the olfactory bulb, which can account for the decrease in the possible decrease in

GnRH neurons, a broad range of differences in pubertal development, and the phenotype of anosmia [24].

Prokineticin-2 (*Prok2*) is a secreted protein that acts upon the receptors prokineticin receptor 1 and prokineticin receptor 2. This protein is very important in the olfaction and has been implicated in olfactory bulb morphogenesis as well as maintaining a healthy reproductive system [10]. The disruption in the olfactory bulb neurogenesis can result in improper GnRH neuron development [20]. It was also found that mice that were null mutants for Prok2 had a reduction in GnRH neurons after they crossed the cribiform plate at E13.5 because the neurons became stuck in tangled olfactory projections [20]. A heterozygous loss of this genes exhibits symptoms similar to that seen in Kallmann's patients and a homozygous loss of the gene results in an IHH phenotype [10]. Since Prok2 is seen to be essential in the development of GnRH neurons and is also implicated for proper migration of these neurons out of the nose, we decided to study this gene in both the *Six3HET* and *Six6KO* mouse models.

Orthodenticle homeobox 2 (*Otx2*) is a type of homeodomain protein that is needed for normal development of the forebrain and has been seen to be expressed in GnRH neurons [25]. Deletion of the *Otx2* gene within GnRH neurons causes a decrease in GnRH neurons present in the hypothalamus and infertility, which can also be consistent with IHH. We decided to examine the presence of this gene in both mouse models because of its role in development.

Ventral Anterior Homeobox1 (*Vax1*) is a homeodomain protein that has been implicated in regulating the HPG axis [26]. When mice are heterozygous for the *Vax1* gene, there is a decrease in *Vax1* mRNA levels, which correlates with a decrease in GnRH transcript. This ultimately resulted in a subfertility for both the male and female mice.

1.2 Results

Gene expression was examined in the adult hypothalamus of WT and *Six3*HET mouse models and was used as a marker for the expression levels that may be present during embryogenesis. A trend towards significance was defined as $p < 0.1$. From our data, we found that *Nelf* and *Npn2* are factors that have expression levels that are trending towards a decrease (Figure 6a,b).

Npn2, an axonal guidance factor, had a trend towards a decrease in mRNA expression (Figure 6b). *Nelf*, a guidance factor for olfactory projections, also had a trend towards a decrease in mRNA expression. A trend towards a decrease in *Npn2* and *Nelf* is consistent with our hypothesis that there is a lack of factors to aid GnRH neurons along their migratory path, which may be why the neurons never reach the hypothalamus.

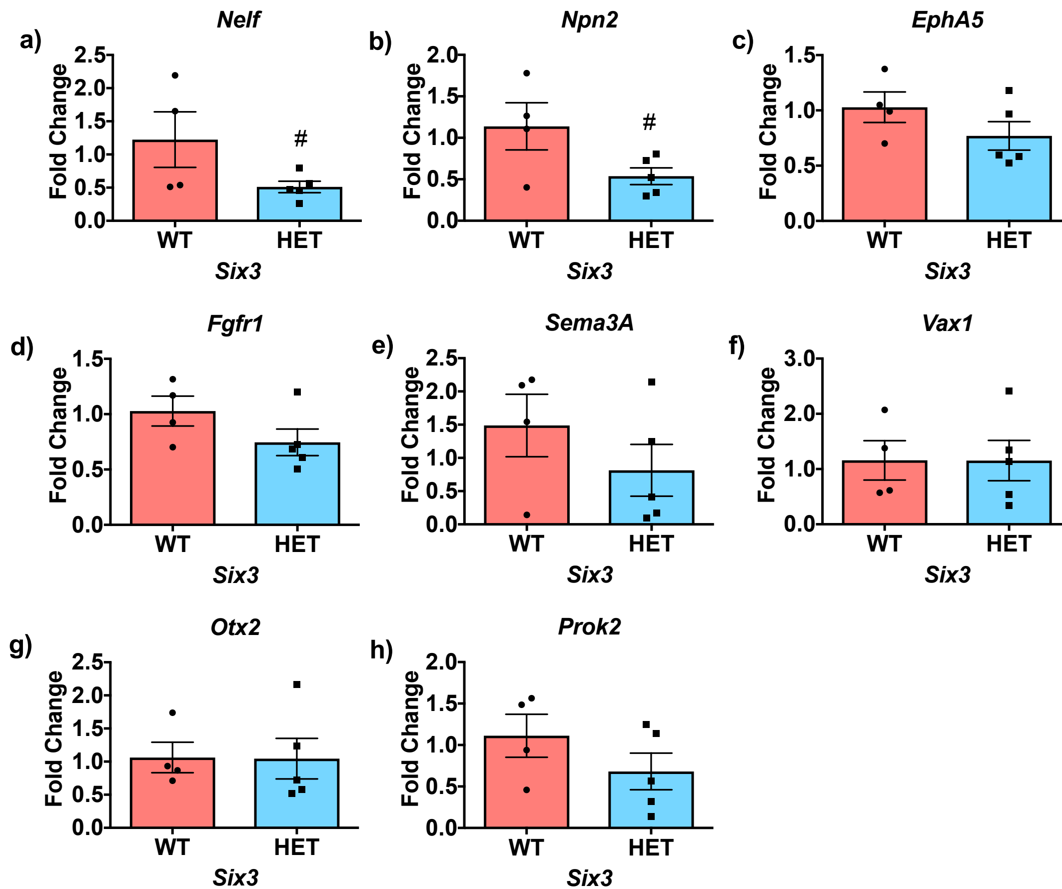

Figure 6: mRNA expression of migratory genes via qRT-PCR. Adult hypothalamic micropunches from wild-type (WT) and *Six3* heterozygous (HET) mice were analyzed. Expression from genes was analyzed using $2^{-\Delta\Delta Ct}$ and normalized to the housekeeping gene *H2afz*. Data are presented as fold change relative to WT. Statistical analyses were performed using a student's t-test (WT n = 4, HET n = 5). # indicates trending towards significance, with a trend indicated by $p<0.1$. a) *Nelf* mRNA shows a trend towards a decrease expression in HETs vs. WTs, $p = 0.1$. b) *Npn2* mRNA shows a trend towards a decrease in expression in HETs vs WTs, $p = 0.065$. c) No significant difference seen between HET and WT *EphA5* mRNA expression. d) No significant difference seen between HET and WT *Fgfr1* mRNA expression. e) No significant difference seen between HET and WT *Sema3A* mRNA expression. f) No significant difference seen between HET and WT *Vax* mRNA expression. g) No significant difference seen between HET and WT *Otx2* mRNA expression. h) No significant difference seen between HET and WT *Prok2* mRNA expression.

Since qRT-PCR detected a trend towards a decrease in expression for the genes *Npn2* and *Nelf*, we wanted to use immunohistochemistry (IHC) to investigate the presence of these factors along the migratory pathway of GnRH neurons during embryogenesis (E14.5). We chose to look

at E14.5 because this is near the peak of GnRH neuronal migration, therefore we expected that if there were a difference in protein expression, that it would be most significant at this time point.

To see how protein expression of *Npn2* differed along the migratory pathway during embryogenesis, we used IHC to examine protein levels of NPN2 and NPN1 at E14.5. NPN2 and NPN1 showed augmented expression by visual inspection in the *Six3*HET mice, with *Six3*HET mice showing no expression of NPN2 (Figure 7a). More specifically, there is staining for NPN2 along the olfactory projections and within the olfactory epithelium. *Npn1* is another gene that is within the same family as *Nnp2* and is also implicated in axonal guidance. IHC revealed that NPN1 expression was much lower in the olfactory bulb and olfactory epithelium of the *Six3*HET mice in comparison to the WT mice, though the HET mice still had slight expression (Figure 7b). These findings may indicate that NPN1 is an additional guidance factor required for GnRH neurons to properly migrate out of the olfactory epithelium and into the hypothalamus. Additionally, these data imply that NPN1 cannot take over in the absence of NPN2 in GnRH migration.

IHC for NELF was completed on embryos at E14.5 because this is the peak of neuronal migration. NELF expression was visually observed and not seen to be different between WT and *Six3*HET mice. Both genotypes of mice had strong NELF expression in the cribriform plate.

The results from qRT-PCR revealed no trend in mRNA expression for the genes of *EphA5*, *Fgfr1*, *Sema3A*, *Vax1*, *Otx2*, and *Prok2*. While there was no difference detected in the expression levels of these genes in the adult hypothalamus, we cannot conclude that they are unaffected by the reduction of *Six3* during GnRH migration.

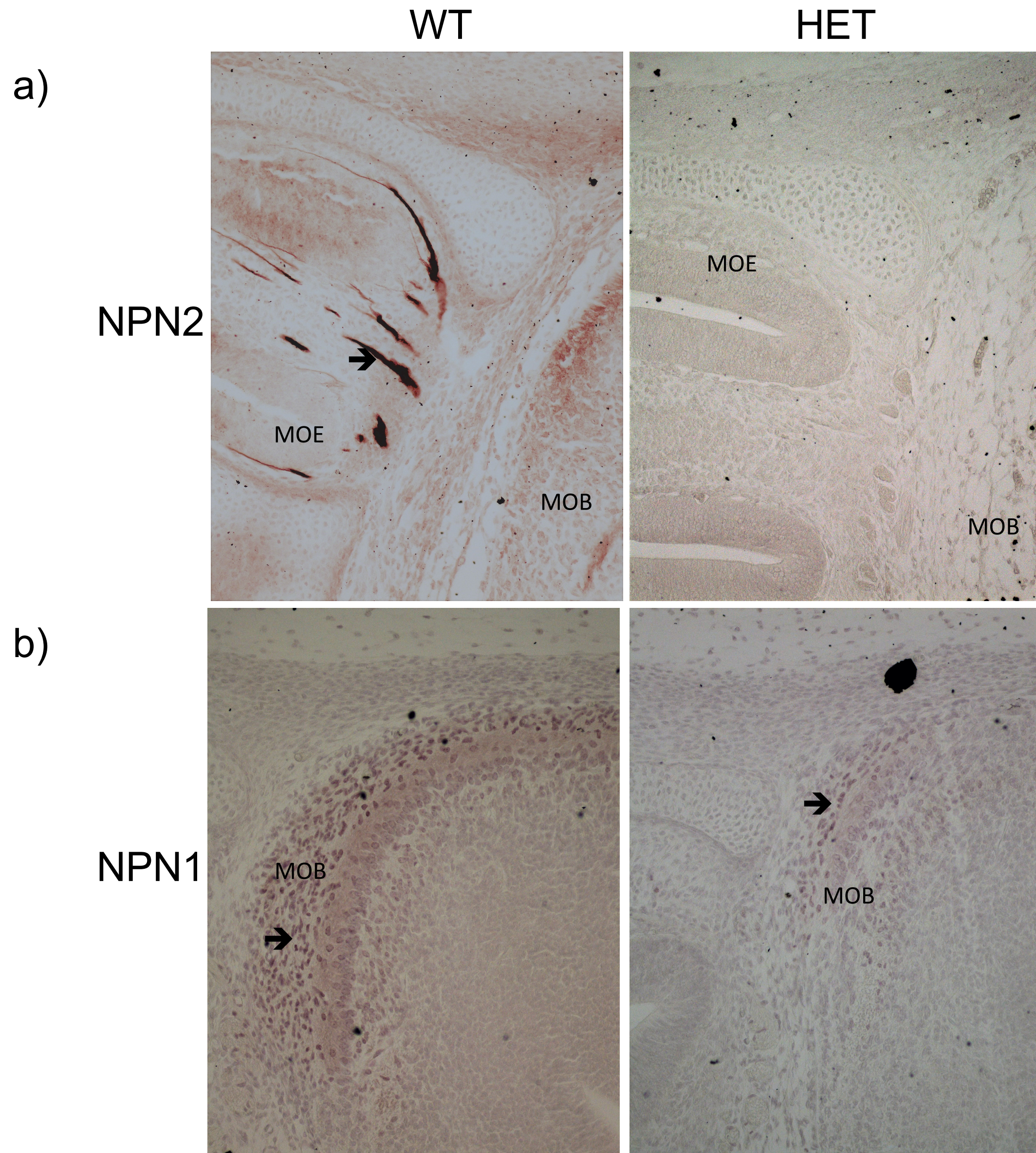

Figure 7: Immunohistochemistry for NPN2 and NPN1 in E14.5 WT versus *Six3*HET mice. Main olfactory epithelium (MOE) and main olfactory bulb (MOB) indicated. Arrows are indicating areas with high expression. a) NPN2 expression. Arrow showing high expression of NPN2 along the olfactory projections in the MOE. Absence of expression in *Six3*HET mice. b) NPN1 expression strongest in the MOB, with decreased expression in *Six3*HET mice.

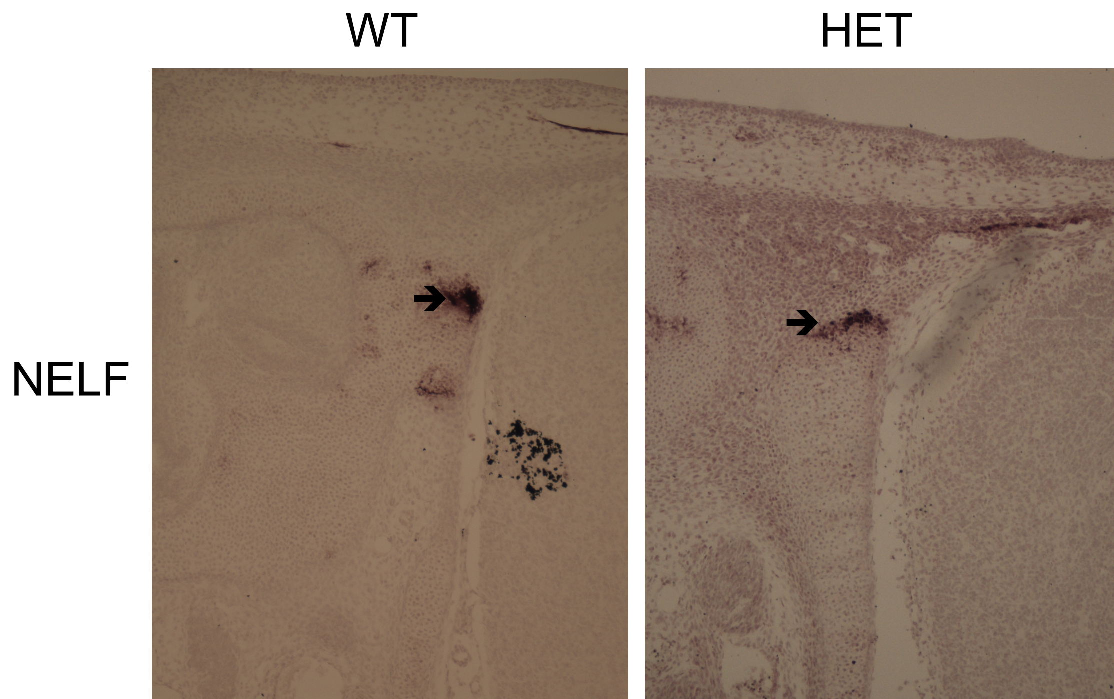

Figure 8: Immunohistochemistry for NELF in E14.5 WT versus *Six3*HET mice. Arrows are indicating areas with high expression. Staining seen in cribriform plate. No perceived difference between expression in WT versus *Six3*HET.

1.3 Discussion

In the *Six3*HET model, many GnRH neurons are halted within the olfactory placode but the mechanism behind this phenomenon has yet to be fully characterized. As a result, we investigated factors that are associated with axonal guidance of GnRH neurons and olfactory projections.

Npn2 showed a decrease in mRNA expression in the adult hypothalamus as well as a decrease in protein expression in E14.5 *Six3*HET embryos along the olfactory projections as well as within the olfactory epithelium (Figure 6b and Figure 7a). These data further support the idea

that *Npn2* is needed for proper development of the olfactory projections that are needed to transport the GnRH neurons out of the nasal cavity. Additionally, in mice that have a knock-out of Npn2, its loss causes defasciculation of the olfactory nerves, a reduction in fertility, and decreased number of GnRH neurons within the hypothalamus [27]. Therefore, lack of this protein could be responsible for the halt in migration of GnRH neurons that is witnessed in *Six3*HET mice.

We also observed a reduction in NPN1 protein expression in *Six3*HET mice (Figure 7b). This could indicate that NPN1 is also needed for the guidance of the GnRH neurons out of the nasal cavity, but because it is so strongly expressed in the olfactory bulb, it might also be implicated in guiding the GnRH neurons further along the migratory route within the brain. *Npn1* has also been seen to be expressed in sensory neurons within the main and accessory olfactory epithelia [18, 27]. Immunofluorescence staining has shown that immortalized immature GnRH neurons, Gn11s, express *Npn1* [18]. In GnRH migration assays, it was noted that *Npn1* and *Npn2* are needed in order for VEGF to have a positive effect on GnRH neuronal migration when semaphorins (like Sema3A) are acting as a negative cues for GnRH neuronal migration [18]. Therefore, it is possible that the decrease of *Npn2* and *Npn1* within *Six3*HET mice is preventing the GnRH neurons from exiting the nasal cavity. The lack of the expression of these genes could also cause GnRH neurons to be found off of their migratory path or halt their migration because of negative cues inhibiting their journey.

From the IHC staining, we visually observed high staining of NPN2 along the olfactory projections, but we did not see any staining of olfactory projections in the *Six3*HET mice. This finding brings up the question of the number of olfactory projections are present relative to WT mice. It is known that some of the GnRH neurons are able to migrate out of the olfactory placode, so this indicates that there are some olfactory projections present. Staining for the structural

presence of these olfactory projections could allow us to see if there is a decrease in the number projections or if some of the projections have undergone defasciculation.

Nelf mRNA expression was also decreased in *Six3*HET mice (Figure 6a). The decrease in the mRNA expression of *Nelf* was extremely interesting because *Nelf* is needed for the guidance of olfactory projection [28]. Axons from the olfactory pit and axon bundles that are positive for neural cell adhesion molecules will fasciculate together and allow GnRH neurons to cross through the cribriform plate [28]. In previous studies immunohistochemistry of developing embryos has revealed that NELF is found in the forebrain, olfactory epithelium and olfactory pit [28]. It has also been shown that NELF is expressed in both olfactory sensory cells and GnRH cells during development. Therefore, it is possible that GnRH neurons are halted in the olfactory placode is because of the decrease in olfactory projections caused by a reduction in *Nelf* expression.

Despite the finding that mRNA expression of *Nelf* was decreased in *Six3*HET mice, IHC staining in WT and *Six3*HET E14.5 mice did not appear to be visually different (Figure 6a,8). This finding indicates that *Nelf* may be differentially expressed in adults and not during embryogenesis. Therefore, this finding also may indicate that *Nelf* might not be a contributing factor to mismigration of GnRH neurons in *Six3*HET mice. To follow up these studies, we could examine if *Nelf* mRNA or protein expression differed at any other point during embryogenesis.

We saw no change in mRNA expression levels of the developmental genes *Otx2*, *Prok2*, or *Vax1* (Figure 6f,g,h). We were surprised that altering the *Six3* gene does not affect the levels of the *Vax1* gene; *Vax1*KO mice are seen to have a ~50% reduction of GnRH neurons, though like the *Six3*HET mice, they have normal counts of GnRH neurons within the olfactory placode at the point when GnRH neurons originate [29]. Our finding may indicate that the GnRH neurons developing normally, but that they are unable to reach the hypothalamus because of a different factor, like lack of Npn2, which could cause significantly fewer olfactory projections, which would halt GnRH

neuron migration. We also observed no change in EphA5 and Sema3A mRNA expression (Figure 6c,e). These results were surprising based on the evidence from EphA5 knockout mice, which causes a similar clustering of GnRH neurons within the nose as what is seen in *Six3*HET mice [22].

CHAPTER 2. Factors associated with the apoptotic loss of GnRH neurons in *Six6*KO mice

2.1 Introduction

*Six6*KO mice have approximately 89% loss of GnRH neurons due to apoptosis. Therefore, to elucidate the mechanism behind this phenotype, we decided to investigate the expression patterns of genes that are known to be involved in apoptosis. We examined the genes *Otx2*, *Dach1*, *Ark*, *p27kip1* and *Prok2*, which are all genes that have an effect on apoptosis or development of GnRH neurons. *Otx2* and *Prok2* were previously described in Chapter I (p. 14).

Factors associated with apoptosis

Dachshund 1 (*Dach1*) has been seen to have a role in proliferative processes, specifically with that associated with the eye and in pituitary cells [30]. *Dach1* and *Six6* work together to regulate proliferation through the repression of cyclin dependent kinase inhibitors. One of these inhibitors is *p27kip1*, which specifically acts to inhibit proliferation in retinal cells [31]. It is thought that over-expression of this cyclin dependent kinase inhibitor can lead to conditions like microopthalmia, seen in *Six6*KO mice. Since *Six6* works to directly repress *p27kip1*, it is understandable that overexpression of Dach1 can lead to overdevelopment of eyes because *p27kip1* would be very inhibited. We are interested in whether or not *p27kip1* is also having an effect of the proliferation of GnRH neurons, because it is also regulated by *Six6* binding.

Adhesion related kinase (*Ark*) helps to prevent programmed cell death in the extracellular signal-regulated kinase (Erk) and the phosphoinositide 3-kinase (Pi3-K) pathway. *Ark* expression is seen in Gn10 immortalized embryonic GnRH neurons derived from the olfactory area of the mice during GnRH migration, but no expression is seen in post-migration adult GnRH neurons (GT1-7) [32]. Therefore, we hypothesize that it may also have an anti-apoptotic role in GnRH neurons.

2.2 Results

qRT-PCR analysis showed no trend towards significance in either upregulation or down regulation of the genes *Ark*, *Dach1*, *p27kip1*, *Otx2*, or *Prok2* (Figure 9 a-e). However, the findings of *Dach1* were compelling; while the data are variable, some of the *Six6*KO mice had a 4-fold or 8-fold change relative to the WT (Figure 9b).

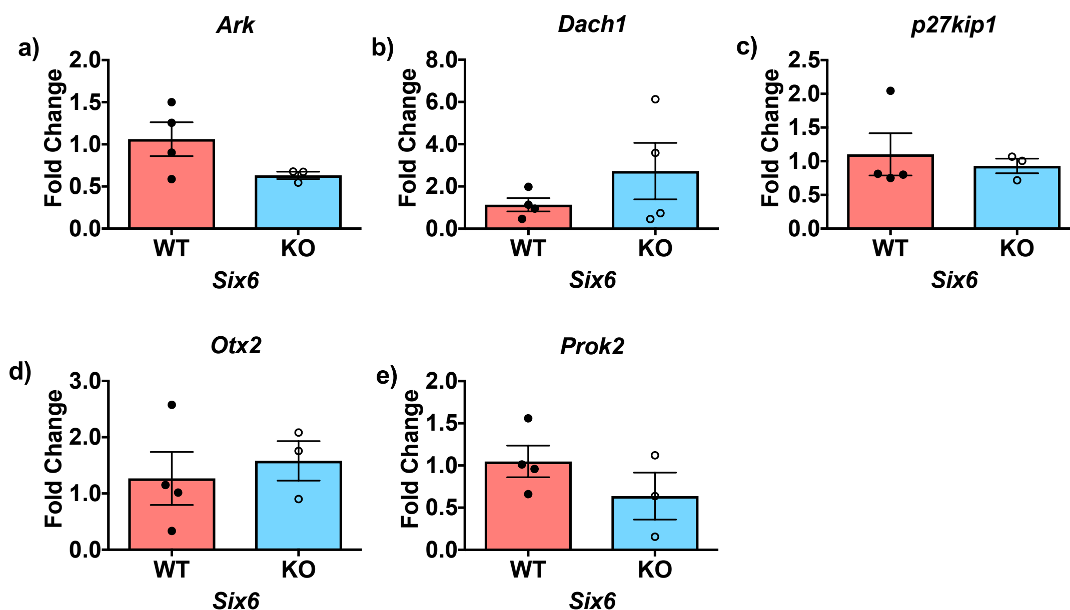

Figure 9: mRNA expression of migratory genes via qRT-PCR. Adult hypothalamic micropunches from wild-type (WT) and *Six6* knock-out (KO) mice were analyzed. Expression from genes was analyzed using $2^{-\Delta\Delta Ct}$ and normalized to the housekeeping gene *H2afz*. Data are presented as fold change relative to WT. Statistical analyses were performed using a student's t-test (WT n=4, HET n=3-4). a) No significance between KO and WT *Ark* mRNA expression, b) No significance between KO and WT *Dach1* mRNA expression, p=0.29, c) No significance between KO and WT *p27kip1* mRNA expression, d) No significance between KO and WT *Otx2* mRNA expression, e) No significance between KO and WT *Prok2* mRNA expression.

Dach1 will bind with Six6 in order to repress cyclin dependent kinases, like *p27kip1* [33]. As stated previously, *Six6* has a significant role in eye development and it is seen in *Six6*KO mice

that there is a significant upregulation of cyclin-dependent kinases including *p27kip1*, which can cause cells to prematurely exit the cell cycle [33]. Expression of these factors had been studied in the eye, but it's possible that this interaction could be affecting the survival of GnRH neurons as well.

2.3 Discussion

The reason for the extreme loss of GnRH neurons in *Six6*KO mice is still unknown, but we hypothesize that it is occurring because of the increase of apoptotic factors or the decrease of developmental factors. As a result, we investigated the various apoptotic and developmental factors to determine if they might be implicated in the loss of these GnRH neurons.

A trend toward an increase in *Dach1* was increased in the *Six6*KO mice (Figure 9b). Normally *Six6* will bind to *Dach1*, to repress cyclin dependent kinases [33]. It is possible that the lack of *Six6* results in an abundance of *Dach1*. This is because *Six6* typically binds to *Dach1* to inhibit proliferation inhibitors. The absence of *Six6* could cause an influx of inhibitors, which could result in an increase in *Dach1*. *Dach1* may be trying to compensate for the loss of *Six6* in order to allow for proliferation to still occur. There may also be other cyclin-dependent kinase inhibitors, other than *p27kip1*, that are upregulated causing the increase in apoptosis.

*Six6*KO had no significant change in *Ark* mRNA expression levels (Figure 9a), indicating that the apoptosis may be acting along a different pathway. We expected *p27kip1* to be upregulated because it is a known factor to inhibit proliferation, and it is also repressed when *Six6* and *Dach1* are bound to it; however, we found there to be no change in mRNA expression (Figure 9c). This could indicate that there are other factors that are lacking, which might be needed to bind to inhibitors of proliferation.

We also observed no change in mRNA expression of the developmental factors *Otx2* and *Prok2* in *Six6*KO mice (Figure 9d,e). These data could indicate that the development of the GnRH neurons is unaffected, which would be consistent with their ability to migrate out of the nose, but instead there is an apoptotic pathway that is active causing there to be a significant reduction of GnRH neurons within the hypothalamus.

GENERAL DISCUSSION

Infertility can be caused by a myriad of factors, some of which are caused by abnormal hormone levels. The hypothalamic-pituitary-gonadal (HPG) axis is critical in maintaining healthy fertility. In the hypothalamus gonadotropin-releasing hormone (GnRH) is secreted from GnRH neurons. GnRH then then acts upon the anterior pituitary to stimulate the secretion of gonadotropins. These gonadotropins will then stimulate the secretion of estrogen, progesterone, and testosterone from the gonads. Defects at any level in this cascade affect fertility. Alterations to the levels of GnRH can result in disruptions within the HPG axis. Two genetic conditions that can be associated with alterations in the level of GnRH are Idiopathic Hypogonadotropic Hypogonadism and Kallmann's Syndrome. Both of these genetic conditions are rare and many are of unknown genetic cause.

Six3 and *Six6* are two genes that are implicated in GnRH neuronal loss, though their mechanisms for the loss of neurons is currently unknown. GnRH neurons are unique in that migration from the nose to the brain is necessary for its function. Variance in the level of factors that are present in the environment along this migratory pathway are thought to play a role in GnRH neuronal loss of both the *Six3* and *Six6* mouse models. We found that there may be a decrease in *Npn2*, *Npn1*, and *Nelf* expression in the *Six3*HET mouse model, which we feel is contributing to the mismigration of GnRH neurons. *Six6*KO mice may have an increase in *Dach1*, which is needed for the cell cycle to occur. While we theorized that Dach1 would decrease because of the significant amount of apoptotic loss within GnRH neurons, this also opens up the idea that another pathway might be present that is causing the loss of GnRH neurons or maybe a protective mechanism is causing the upregulation of Dach1.

We performed qRT-PCR on adult hypothalamic micropunches to give us data that would be representative of what was occurring during embryogenesis. Although, due to the fact that these genes are needed for axonal migration and development, it is possible that these genes are expressed at different levels during embryogenesis and that they may be affected by different processes that are occurring during development. This could result in an inaccurate depiction of the expression of various factors during embryogenesis. While an experiment was attempted in embryos (E14.5), the variation between samples was too great to interpret the data. This was likely due to the inability to provide precise punches in embryos that small. Imprecision was confirmed by highly variable qRT-PCR data. Micropunch accuracy is likely one of the causes for the high variability amongst the qRT-PCR data. In addition, mouse work is also highly variable and the reduced fertility in the mouse models compromised our ability to achieve a higher number of subjects for each condition. Power analysis could be done to determine the correct number of animals that would be needed to achieve significance in for all of the factors that were trending towards significance.

To further understand the mechanism by which these factors are affecting the migration and apoptosis of GnRH neurons, it would be interesting to further study the temporal and spatial differences of the factors at different time points during embryogenesis. Immunohistochemistry could allow us to determine if there is a variance in the protein expression level along the migratory path of GnRH neurons throughout the course of migration. We could also determine if there is a specific time point in which the factors are either upregulated or downregulated. Additionally, we could examine the expression levels of different factors. Since *Six6*KO mice begin with normal GnRH neuron counts and the GnRH neurons are lost by E17, we theorize that there is a strong likelihood of an apoptotic factor being upregulated in the *Six6*KO model. Overall, we have provided insight upon the mechanism by which GnRH neurons are lost as the result of genetic variances of the genes *Six3* and *Six6*.

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