CAROLINE CAETANO DA SILVA

O *nr2e1* influencia o comportamento exploratório, mas não é necessário para a diferenciação hormonal hipofisária no zebrafish (*Danio rerio*).

Dissertação apresentada à Faculdade de
Medicina da Universidade de São Paulo para
obtenção do título de Mestre em Ciências

Programa de Endocrinologia

Orientadora: Dra. Luciani Renata Silveira de Carvalho

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Name: SILVA, Caroline Caetano da

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RESUMO

SILVA, Caroline Caetano da. **O *nr2e1* influencia o comportamento exploratório, mas não é necessário para a diferenciação hormonal hipofisária no zebrafish (*Danio rerio*)**. 2017, Dissertação (mestrado em Ciências) – Faculdade de medicina, Universidade de São Paulo, São Paulo, 2017.

Hipopituitarismo congênito é caracterizado por deficiência hormonal múltipla devido a mutações de fatores de transcrição envolvidos na embriogênese hipofisária. As células-tronco estão presentes na hipófise e são caracterizadas por dar origem a uma célula progenitora e uma célula indiferenciada por divisão assimétrica. Estão envolvidas na hipófise em processos de alta demanda metabólica em diferentes fases da vida. Em estudos prévios, observou-se o acúmulo dos marcadores de células-tronco *Sox2* e *Nr2e1* no camundongo Ames, que apresenta mutação no gene *Prop1*. O *Sox2* é o marcador consenso de células-tronco na hipófise enquanto que o *Nr2e1*, nunca antes caracterizado na hipófise, é essencial para a manutenção de células-tronco e neogênese no cérebro. A perda de função deste gene pode causar agressão e falta de instinto materno em camundongos. Com isso, o objetivo desse projeto foi utilizar o animal modelo zebrafish para avaliar o papel repressor do gene *prop1* e caracterizar o gene *nr2e1* bem como, confirmar se o mesmo está envolvido com a diferenciação terminal na hipófise, e sua interferência no comportamento do animal mutado. O zebrafish se encaixa adequadamente nesse projeto pois é de fácil manutenção, econômico e com rápido desenvolvimento. No presente estudo criou-se 2 modelos de zebrafish utilizando-se a técnica de edição genômica conhecida como CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) para nocautear os genes *prop1* e *nr2e1*. Esta técnica permite uma interrupção específica e substituição de bases no genoma, resultando em uma alta especificidade, baixa toxicidade celular e é herdável. O zebrafish homozigoto com mutação no gene *nr2e1* se desenvolve e reproduz como o animal controle, porém apresenta um comportamento mais exploratório quando comparado com o animal selvagem e o heterozigoto. A imunofluorescência para o anticorpo *Sox2* no animal mutado mostrou se diferente do selvagem, pois apresenta um aumento da expressão temporal e o mesmo não se colocaliza com o *Nr2e1*. A imunofluorescência feita com os hormônios não se mostrou diferente entre o mutado e o selvagem. Conclui-se diante dos achados de normalidade do desenvolvimento, fertilidade, ausência de co-localização com o gene *Sox2* e presença de hormônios como *Tsh*, *Fsh* e *Gh*, que o gene *nr2e1* não é crucial na diferenciação terminal na hipófise porém o animal mutado apresenta um comportamento diferente do animal selvagem. Os resultados da caracterização do zebrafish com mutação no gene *prop1* ainda estão em andamento devido a dificuldade de se estabelecer essa linhagem.

Palavras-chave: Peixe zebra, Sistemas CRISPR-Cas, hipopituitarismo, Imunofluorescência, Células-tronco, Hormônios.

ABSTRACT

SILVA, Caroline Caetano da. ***nr2e1* influences exploratory behavior but is not necessary for terminal hormone differentiation in the zebrafish (*Danio rerio*) pituitary.** 2017, Dissertation (Master of Science) – Faculdade de medicina, Universidade de São Paulo, São Paulo, 2017.

Congenital hypopituitarism is characterized by multiple hormone deficiencies due to mutations in transcription factors involved in pituitary embryogenesis. Stem cells, which by definition can each give rise to a progenitor and an undifferentiated cell by asymmetric division, are present in the pituitary gland and are important during periods of high metabolic demand in different phases of life. In previous studies, the accumulation of the stem cell markers Sox2 and Nr2e1 was observed in the Ames mouse, which harbors a mutation in *Prop1*. Sox2 is the consensus stem cell marker in the pituitary gland, while the role of Nr2e1 in the pituitary development has not been characterized although it is essential for neural stem cell maintenance and neogenesis in the brain and its loss of function causes pathological aggression and lack of maternal instinct in mice. In this project, the zebrafish animal model was used to characterize the role of *nr2e1*, to confirm whether this gene can be involved in the pituitary terminal differentiation, and to determine the effects of this gene on animal behavior. The zebrafish is a particularly appropriate model for use in this project because it is easy to maintain, is economical, and has a rapid metabolism and growth rate. In the present study, we created 2 zebrafish models by knocking out *prop1* and *nr2e1* using the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) genome-editing technique. This technique enables highly specific gene/reading frame interruption and/or base substitution in the genome, with low cellular toxicity and high heritability. Zebrafish with homozygous *nr2e1* mutations develop and reproduce similarly to wild-type zebrafish, but present a more exploratory behavioral pattern compared to wild-type and heterozygous zebrafish. Based on immunofluorescence, Sox2 expression was higher in the mutant zebrafish than in the wild type and was not co-localized with Nr2e1 expression. Hormone expression did not differ between wild-type and mutant zebrafish. We conclude that *nr2e1* is not crucial in the terminal differentiation of the hormone-forming pituitary gland; however, it induces a distinct behavioral phenotype at the larval stage. Analyses of zebrafish harboring a *prop1* mutation are ongoing owing to issues with the establishment of the lineage.

Key words: Zebrafish, CRISPR-Cas systems, Hypopituitarism, Fluorescent technique, Stem cells, Hormones.

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ABBREVIATIONS LIST

CRISPR - Clustered regularly interspaced short palindromic repeats

Cas9 - CRISPR associated protein 9

Nr2e1 - Nuclear receptor subfamily 2 group E member 1

Prop1 - homeobox protein prophet of Pit-1

Sox 2- SRY (sex determining region Y)-box 2

Oct4 - octamer-binding transcription factor 4

Nestin - neuroectodermal stem cell marker

Hpf – hours post fertilization

Dpf – days post fertilization

Gh - Growth hormone

Lh- Luteinizing hormone

Fsh- Follicle-stimulating hormone

Tsh - Thyroid-stimulating hormone

Prl – Prolactin

Acth - Adrenocorticotrophic hormone

F0 -Founder fish

F1- filial 1, offspring of F0

DNA- deoxyribonucleic acid

RNA - ribonucleic acid

sgRNA – single guide RNA

PCR- Polymerase chain reaction

dNTP - Deoxyribonucleotide triphosphate

Wt – Wild type

Mut – Mutant

For zebrafish the current nomenclature guidelines are updates to rules established during a discussion session at a meeting in Ringberg, Germany, in March 1992, and are widely accepted by most zebrafish labs.

Genes: Full gene names are lowercase italic

Protein: The protein symbol is the same as the gene symbol, but non-italic and the first letter is uppercase.

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1. INTRODUCTION

1.1 The Pituitary

The pituitary gland is an important organ that produces and stores hormones that regulate homeostasis. The posterior lobe, which is known as the neurohypophysis, originates from the neuroectoderm, while the anterior and intermediate lobes, known as the adenohypophysis, are derived from the oral ectoderm. In zebrafish, the development of the pituitary gland is somewhat different, in that the anterior pituitary gland begins to develop directly from the placode in contact with the development of neural tissue [1]. However, both the neurohypophysis and adenohypophysis have the following five types of endocrine cells: somatotrophs that produce growth hormone (GH), mammothrophs that produce prolactin (PRL), thyrotrophs that produce thyroid stimulating hormone (TSH), gonadotrophs that produce luteinizing hormone (LH), follicle stimulating hormone (FSH), and finally, corticotrophs that produce adrenocorticotrophic hormone (ACTH) [2]. In addition to endocrine cells, the presence of stem cells in the pituitary gland has also been known since 1969 when Yoshimura et al. [3] transplanted pituitary cells, which subsequently led to hormone-secreting action, in rats that were hypophysectomized. The pituitary gland has the ability to adapt the absolute and relative numbers of each cell subtype in response to different metabolic demands, such as growth, puberty, gestation, and lactation [4]. Although the exact mechanisms responsible for these changes are not well known, it is thought that mitosis of the pre-existing cells, transdifferentiation (conversion between phenotypes), and differentiation of stem cells all play a role [5-7].

Despite conflicting results from studies about the best progenitor/stem cell markers specific for each tissue, it is known that these markers are of great value for understanding cell differentiation. The most commonly used stem/progenitor markers are as follows: SRY-

box containing gene 2 (Sox2), Oct4 (Pou5f1), Nestin, Nanog, CD44, and nuclear receptor subfamily 2 group E member 1 (Nr2e1) [8-13].

1.2 Stem Cells and Progenitor Cells in the Pituitary

Stem cells are characterized by their capacity for proliferation, self-renewal, and differentiation potential. They have already been described in cell renewal and homeostatic regulation of the pituitary [14]. Stem cells, by definition, have the self-renewal capacity involved in turnover, plasticity, or repair [15, 16]. By asymmetric division, one stem cell must be able to generate another stem cell, as well as a progenitor cell [16].

Progenitor cells may not have the ability, or have a limited ability, to self-renew, but they may be able to proliferate to form a pool of cells that will enter the tissue differentiation program [16, 17]. Thus, stem cells in the pituitary are important for the maintenance of the endocrine cells that are necessary for homeostasis.

In mice, the stem cell niche may be in the marginal zone around the lumen of Rathke's pouch, between the anterior and intermediate lobes of the pituitary, because cells in this region are believed to generate all six pituitary hormone cell lineages. From a therapeutic viewpoint, the ability to cultivate and elicit stem cell growth in the pituitary during the pre-differentiation state could be helpful for the long-term treatment of pituitary deficiencies.

1.3 Hypopituitarism

Hypopituitarism is characterized by the decreased production of one or more pituitary hormones, which may occur during hypoplasia and dysmorphology. In humans, mutations in the *PROPI* gene are the most common causes of congenital hypopituitarism, which is manifested by the variable and progressive deficits in GH, TSH, PRL, ACTH, and gonadotrophins such as FSH and LH [18-20].

The PROP Paired-Like Homeobox 1 (*PROP1*) gene has three highly conserved exons, located on chromosome 5q35.3 that encodes 226 amino acids, which retains deoxyribonucleic acid (DNA) binding ability and transcriptional activation [21]. The majority of mutations that elicit congenital hypopituitarism are located within the second exon of *PROP1*. *PROP1* is required to initiate the expression of POU Class 1 Homeobox 1, which is also known as *POU1F1*. However, it also plays an important inhibitory role on the regulation of the expression of the Homeobox expressed in ES cells 1 (*HESX1*) protein. Therefore, *PROP1* can either act as a transcriptional activator or repressor factor, depending on the associated cofactors. Genetic studies have shown that the temporal regulation of *PROP1* expression is critical for the normal development of the pituitary gland. Its premature expression in the Rathke's pouch leads to agenesis of the gland, probably due to the inhibition of *HESX1* expression [22].

PROP1 is responsible for the ventral migration of progenitor cells, i.e., from the proliferative zone of the Rathke's pouch into the anterior lobe, and for their differentiation. This migration is due to the process of mesenchymal-epithelial differentiation [23]. Failure in the ventral migration of progenitor cells due to mutations, can consequently lead to a lack of differentiation that results in pituitary dysmorphology with decreased adenohypophysis, but with a pituitary volume within the normal range at birth. Most individuals with hypopituitarism had a normal birth weight and size [24], but had postnatal growth retardation, combined with hormonal deficiencies [18, 25].

1.4 Sox2

Progenitor cells in the pituitary are also able to give rise to endocrine cells [14]. *SOX2*, along with *SOX1* and *SOX3*, are clustered in the B1 subfamily of the *Sox* gene, and are involved in several stages of embryonic development and cell differentiation, playing central

roles in embryogenesis [26]. During pituitary development, SOX2 is expressed at the beginning of Rathke's pouch formation and is maintained until the process of terminal cell differentiation occurs [27]. *SOX2* has several mutations, which has already been described in humans and in animals [27]. Its expression, as a central marker of undifferentiated cells (stem cells and/or progenitor cells), is maintained in the zone of the marginal layer of the pituitary, where it supplies the glandular metabolic demand as it is essential for normal development and hypothalamic-pituitary function. [28, 29]

1.5 Stem Cells and Progenitor Cells in Animal Models of Hypopituitarism

Although it is known that stem cells play a role in pituitary renewal during major periods of cellular and metabolic demand such as puberty, gestation, and lactation, little is known about the expression pattern of stem/progenitor cell markers in the pituitary gland of animal models with congenital hypopituitarism. In 2013, Chang [30] demonstrated the increased expression of stem cell markers, such as *SOX1* and *NR2e1*, in the Ames mice, i.e., a *PROP1* mutant, during the postnatal period [31]. It is also known that SOX2 acts as a positive regulator of NR2E1 [32]. NR2E1 has an important function in cerebral neogenesis, but has never been characterized in the pituitary.

Mice that are with a mutation for the *Nr2e1* gene [33] are aggressive and do not copulate easily, which makes it challenging to characterize genes in the pituitary of this animal.

1.6 Zebrafish (*Danio rerio*) as an Animal Model

The use of animal models is crucial to create new drugs and treatments, and understand genetics, endocrinology, and so many other aspects of medicine. Although mice are commonly used for research, but they have a slow reproduction rate, are difficult to handle, and have a high cost associated with their use, compared to zebrafish.

The zebrafish is a small teleost (3–4 cm), which belongs to the Cyprinidae family and is of Asian origin. Although recent, its use as an animal model in research is very advantageous given its easy maintenance, small size, cost-efficient breeding, and fast metabolism. It also has a high reproductive rate and presents an important homology with mammals. Zebrafish constitute an excellent experimental model for behavioral, genetic, and toxicological studies, to unveil the mechanism underlying various human diseases, in addition to testing new therapeutic agents [34, 35].

In the past few years, there has been considerable progress in knowledge about the genetic traits and genomic information of zebrafish [36-38]. The genes of this teleost are evolutionarily conserved and present a high degree of similarity to human and mouse genes [34, 39].

To support studies with this animal model, an information network was created named ZFIN (<http://zfin.org>), in which research laboratories from around the world deposit information about the new zebrafish findings [40]. In addition, there is a frequently updated maintenance manual on the conditions required to raise zebrafish in a laboratory [41].

The characteristics of zebrafish embryogenesis, as well as its development, are well known [42, 43]. Thus, the zebrafish is widely used as an animal model for genetic and endocrinology studies, since its embryonic formation is faster, easier to visualize, and associated with a lower cost, compared to mice. In addition, the zebrafish has a faster life cycle, than mice, with 72 h, 45 days, and 90 days post-fertilization considered as the beginning of the larval stage, juvenile stage and adult stage, respectively. The early developmental stage of the zebrafish is shown in Figure 1.

Figure 1: Early developmental stages of zebrafish. Adapted by UCSB image

1.7 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR Associated Protein 9 (Cas9)

Three methods, which all use specific sequences of endonucleases that break the double strand of DNA, have been developed to generate target mutations in zebrafish. In addition, the three techniques use a low-fidelity non-homologous end joining repair mechanism, resulting in small insertions and deletions (InDel). The use of zinc-finger nucleases (ZFN), with multiple zinc fingers conferring specificity in the sequence [44], was the first technique to emerge; however, it is not frequently used since it is laborious and not very specific. Given its high specificity, which is naturally derived from the binding of DNA domains, the use of transcription activator like effector nucleases (TALENs) rapidly replaced the use of ZFN [45].

However, generating mutations using TALENs is still time consuming. The third genome-editing method, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9), is widely used in different strands. The two components of this system use ribonucleic acid (RNA) to guide the Cas9 endonuclease to the target through the base pairs. Since it is a simple system and guide RNA (gRNA) is easy to draw, this method has become popular worldwide [46].

Figure 2: Genome-editing using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system. Since they carried the mutation, F1 was used for experiments

The CRISPR/Cas system can be extremely efficient in zebrafish; when Cas9 and gRNA are injected into the zygote, it can generate an organism with biallelic inactivation of the gene of interest in all cells [47]. Two lineages of mutant zebrafish are sufficient to understand the role of *Nr2e1* in the pituitary, and evaluate the role this gene plays in social behavior. Further, the *prop1* mutant will help to elucidate the correlation between these two genes of interest and the role of *nr2e1* in the pituitary gland.

2. OBJECTIVES

The primary objective of this project was to produce a heritable *nr2e1* and *prop1* knockout in the zebrafish using CRISPR-Cas9. The secondary aims of this project were to characterize the role *nr2e1* plays in the terminal differentiation of the pituitary gland and to analyze the behavior of the heterozygous and wild type *nr2e1* mutant.

3. MATERIAL AND METHODS

3.1 Zebrafish (*Danio rerio*)

Zebrafish were raised in an Aquatic-Habitats system, using a 14:10-h light-dark cycle at 28°C. Embryos were obtained from natural crossing, and raised according to standard methods. All procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee and the Medical school University of São Paulo CEUA and CIBIO program (n° 046/14). Mutant lineages, from injected fish (F0) were raised to adulthood for analysis. The University of São Paulo aquariums (Tecniplast model) were kept under the following controlled climatic conditions: pH of approximately 7.5; conductivity of 500–800 µs; temperature of 28°C; and a photoperiod of 14 h of light. The animals were fed three times a day with Gemma Micro (Skretting a nutreco company®). We chose to use zebrafish for this project due to the low cost of their maintenance, and their fast development and reproduction.

3.2 Gene Characterization

3.2.1 Samples

Samples were collected at 24, 48, 72, and 96 h post-fertilization, and stored in a freezer at -80°C until the time of RNA extraction for semi-quantitative PCR analysis [48, 49].

3.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

3.3.1 RNA Extraction

Messenger RNA (mRNA) was obtained to analyze the expression analysis of genes using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Total

zebrafish RNA was extracted at different time points. RNA extraction was performed using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. Tissues were lysed and homogenized in the presence of a buffer that contained guanidine thiocyanate (to inactivate the RNases). Following the addition of ethanol to the solution, the RNA molecules acquired the appropriate chemical conditions to bind the column. The material was then treated with the enzyme RNase-Free DNase Set (QIAGEN, Valencia, CA, USA), which prevents contamination with genomic DNA.

Subsequently, the material was washed, eluted in Milli-Q water, and stocked at -80 °C.

The quantification of total RNA extracted was performed using a p300 nanophotometer apparatus (Implen Inc., Westlake Village, CA, USA), with a wavelength of 260 nm.

3.3.2 Synthesis of Complementary DNA (cDNA)

In the current study, a total of 750 ng and 2,000 ng of total RNA were used for complementary DNA (cDNA) synthesis to analyze the gene expression pattern of *nr2e1* and *prop1*, respectively. A high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was used to generate cDNA, according to the manufacturer's instructions. In brief, cDNA was produced using 2 µl of 10× reverse transcription (RT) Buffer, 2 µl of 10× RT Random Primers, 0.8 µl of 25× dNTP Mix (100 mM), and 0.2 µl of Multiscribe® Reverse Transcriptase (50 U/µl). Thermocycler reaction (96 well thermal cycler, Applied Biosystems®) were performed with 25 °C, 37 °C, and 85 °C for 10", 120", and 5", respectively.

3.3.3 Semi-Quantitative Polymerase Chain Reaction

For semi-quantitative PCR, the primers listed in Table 1 were used to quantify gene expression during zebrafish development. As a positive control, we used the gene expression of *Efa1*, as the endogenous gene.

Table 1. Specific primers for amplifying *prop1*, *nr2e1*, *ccnd2a*, *p21*, *sox2* and *efa1*.

Primers	Nucleotide sequences (5'-3')	Expected Fragment Base pairs(PB)
<i>prop1</i> *	(F)5'GGGCGGGTGTTATTAACCCTCACTAAAGAGCTTGCTA GGGTCACCAAA3' (R)5'CCGGGGGGGTGTAATACGACTCACTATAGCCAGTGAGA GCACTGAGGTG3'	375
<i>nr2e1</i> [48]	(F) 5'-TTGGTTTCACAGTTAGTTGC-3' (R) 5'-GGAGAGCAGTCGTGTGATGG-3'	1481
<i>ccnd2a</i> *	(F) 5'- CATGATTGCAACGGGTAGTG -3' (R) 5'- TCCTGCTGTTGCTGTTTTTG -3'	216
<i>p21</i> *	(F) 5'- AAACATCCCGAAAACACCAG -3' (R) 5'- AACGCTGCTACGAGACGAAT -3'	248
<i>sox2</i> *	(F) 5'- GCTCTGCACATGAAGGAACA-3' (R) 5'- TTCATATGCGCGTAGCTGTC -3'	197
<i>efa1</i> [50]	(F) 5'-CGTCTGCCACTTCAGGATG -3' (R) 5'-TGTCTCCAGCCACATTACCA -3'	247

* designed in our laboratory

The reactions for gene fragment amplification were performed in a total volume of 25 µL, with GoTaq® DNA polymerase (Promega®). Primer3 plus software was used to design the primers.

The reactions were placed in a thermal cycler (Applied Biosystems®) and subjected to the cycles outlined in Table 2.

Table 2. Conditions used for gene amplification *prop1*, *nr2e1*, *ccnd2a*, *p21*, *sox2* and *efal*.

Primers	Cycle	Denaturing	Annealing	Polymerization	final Polymerization
<i>prop1</i>	40	94°C - 45''	67°C - 45''	72 °C - 1'	72 °C - 10'
<i>nr2e1</i>	35	94°C - 30'	52°C - 30'	72 °C - 1'	72 °C - 10'
<i>ccnd2</i>	35	94°C - 45''	47°C - 45''	72 °C - 1'	72 °C - 10'
<i>p21</i>	35	94°C - 45''	43°C - 45''	72 °C - 1'	72 °C - 10'
<i>sox2</i>	35	94°C - 45''	51°C - 45''	72 °C - 1'	72 °C - 10'
<i>efal</i>	35	95 °C - 30'	58 °C - 30'	72 °C - 30'	72 °C - 10'

3.4 Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9)

3.4.1 Generation of Cas9 mRNA

To improve protein expression in zebrafish, a zebrafish codon-optimized version of Cas9 (4101 base pair [bp]) was synthesized, and two SV40 large T-antigen nuclear localization signals (NLSs) were added at both amino- and carboxyl-termini to facilitate nuclear entry [51, 52]. The resulting NLS-tagged codon-optimized construct (i.e., NLS-zCas9-NLS) was cloned into a vector under the control of the SP6 or T3 promoter. These two final plasmids (pCS2-NLS-zCas9-8 NLS and pT3TS-NLS-zCas9-NLS, Addgene plasmid ID 47929 and 46757, respectively) were used to generate Cas9 RNA using in vitro transcription. The plasmid was donated by Dr. Wenbiao Chen.

3.4.2 Generation of Single Guide RNA

Cas9, CRISPR RNA (crRNA), and trans-activating crRNA (tracrRNA) are the three main components of the CRISPR/Cas9 system. To further simplify the system, the crRNA and tracrRNA are fused together to form a single chimeric guide RNA (sgRNA) [52-54]. The targets were selected following designing rules for high activity and low off-target effect [53]. The sgRNAs designed and used are shown in Table 3.

Table 3. *prop1* and *nr2e1* sgRNA that were injected in zebrafish (AB) one cell stage

Oligo Name	Bases	Sequence (5' to 3')
prop1 sgRNA T1	60	AATTAATACGACTCACTATAGGACATCTATATCTGACAGGgttttagagctagaaatagc
prop1 sgRNA T2	60	AATTAATACGACTCACTATAGGGTAGTTCGGTGTCTGCGGgttttagagctagaaatagc
prop1 sgRNA T3	60	AATTAATACGACTCACTATAGGAGCTGGAGCACCTGGAGTgttttagagctagaaatagc
nr2e1 sgRNA T1	60	AATTAATACGACTCACTATAGGCAGCACGAGAGGGGACCGgttttagagctagaaatagc
nr2e1 sgRNA T2	60	AATTAATACGACTCACTATAGGGCGGCCCGGGATAGACGgttttagagctagaaatagc

We also designed a cloning-free strategy to streamline the generation of target-specific sgRNA constructs. To this end, two long oligos were synthesized; one was the universal 80-nt oligo (PAGE purified) with the sgRNA crRNA:tracrRNA scaffold as follows: **5'-ttttgcaccgactcggtgccactttttcaagttgataacggactagccttattttaacttgctatttctagctctaaaac-3'**, the other was the 60-nt oligo containing the T7 promoter, the 20-nt gene specific target sequence, and the 20-nt sequence complementary to the 5' part of the crRNA. These two oligos were annealed and filled by the T4 DNA polymerase. The resulting double-stranded DNA served as the template for in vitro transcription to generate the sgRNA. Since there was no cloning involved and only one specific primer was needed for each target, this strategy was time-efficient and cost-effective. It is amenable for large-scale sgRNA synthesis. A similar strategy has been previously used by the Schier lab [55] and other labs. To prepare the sgRNA we followed the protocol outlined by Chen et al [51].

When endonuclease Cas9 was injected, it recognized the protospacer adjacent motif site, i.e., NGG, where it subsequently generated DNA breaks, as outlined in Figure 3.

Figure 3: Single guide RNA (sgRNA) and CRISPR associated protein 9 (Cas9) system. Cas9 recognized the protospacer adjacent motif site (NGG), where it generated a double strand break. Here, using homologous recombination, the DNA will create small insertions and/or deletions (InDels).

3.4.3 Template Design for Knock-in

We tried to create the equivalent mutation of the dwarf Ames mouse through homology directed repair. In the Ames mouse, the spontaneous T to C mutation in *PROPI* resulted in a Serine to Proline substitution at the residue 83, within the alpha1 helix of the homeodomain. We designed the following template to introduce an equivalent mutation into zebrafish *PROPI*:

XhoI

(5'tcCCGCCGCAGACACCGAACTACCTtcagcaatgaACAGCTGGAGCACCT|cGAGcctGctt
tcagacagaaccactatcctgatatctactacagagaggagcttgc3').

This template was injected with the *prop1*-T2 sgRNA, during one cell stage, since it showed the highest mutagenesis efficiency. To analyze the incorporation of the template point

mutation, we amplified the target region and digested the amplicon with the restriction enzyme XhoI (Table 4).

Table 4: Protocol for XhoI enzyme reaction

H ₂ O	7,5µl
CutSmart	1,0µl
XhoI	0,5µl
PCR product	1µl

3.4.4 Generation of Mutant Zebrafish Using the CRISPR/Cas9 Technique

The wild type fish (AB) were left in breeding tanks with dividers in the afternoon, the day before injection. The day after, before removing the dividers for breeding fish, the injection mix was prepared. The 5-µl injection mix included 1–2 µg of Cas9 mRNA, 100–400 ng of sgRNA, 0.5 µl of 0.5% phenol red. Sufficient nuclease-free water was used to make the final volume up to 5 µl. Embryos were collected 10–15 min of natural spawn. Each zygote was injected with 1 nL of injection mix solution. Careful attention was paid to avoid injecting into the yolk.

Figure 4: The zebrafish embryo at the one cell stage

All injected embryos were incubated at 28°C in egg water solution in a 10-cm petri dishes, until their mutagenesis efficiency was analyzed between 1–4 days after the injection.

3.4.4.1 Estimation of Mutagenesis Efficiency in the Injected Embryos (F0)

Before raising the injected embryos to maturity, it was necessary to confirm that a given sgRNA/Cas9 combination had cleaved the target site efficiently. Mutagenesis efficiency can

be estimated by examining the formation of DNA heteroduplexes after amplifying the target region (e.g., 300–500 bp encompassing the cleavage site) from the mutagenized genome using PCR. Heteroduplex DNA forms when the amplicons with the Cas9-induced lesions (usually small InDels) are rehybridized with the wild type or different InDel counterparts. Cas9-induced cleavage efficiency (i.e., the InDel percentage) can then be calculated as the fraction of total DNA. However, given the heterogeneity of lesions in F0, the mutagenesis efficiency is usually underestimated using this approach. The presence of heteroduplexes can be detected more qualitatively by separating the amplicons in a 10% polyacrylamide gel. Heteroduplexes show reduced mobility on the gel, while homoduplexes of InDels can be resolved from the wild type.

3.4.4.2 Isolation of Genomic DNA

At 24 h post-fertilization, three pools of four injected embryos were placed in 0.2 ml Eppendorf tubes, containing 100 µl of 20 mM NaOH. These samples were incubated at 95°C for 20 min, cooled down to 4°C, and neutralized using 20 µl of 1 M Tris-HCl (pH not adjusted).

3.4.4.3 Amplification of the Target Region for Genotyping

Primers were designed to flank the predicted cleavage site in the genome with a predicted product size of 367 bp and 368 bp, for *prop1* and *nr2e1*, respectively. The sequences are outlined in Table 5.

Table 5: Sequence of primers to amplify the interested region.

Oligo Name	Sequence 5' to 3'
prop1 GTF	TTGGGGAAAATCTGTCGCTGT
prop1 GTR	GGTTACCTGTATGCGAGCCT
nr2e1 GTF	AGGCCTATGCAATTATTTTCCAC
nr2e1 GTR	CAAGTCCCACAATTGCCTGC

3.4.5 Polymerase Chain Reaction (PCR)

A typical 3-step PCR is usually effective in amplifying most genomic targets. However, the PCR conditions for different targets may need to be optimized. A typical 25- μ l PCR setup was as follow: 2.5 μ l of 10 $\times$ PCR buffer, 3 μ l of 25 mM MgCl₂, 0.5 μ l of 10 mM dNTPs, 0.5 μ l of 10 μ M forward primer, 0.5 μ l of 10 μ M reverse primer, 2 μ l of genomic DNA preparation, 0.2 μ l of Taq DNA polymerase (5 U/ μ l), and 15.8 μ l of H₂O. The conditions of the PCR program was as follows: 94°C for 2 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. A total of 2 μ l of the PCR product was run on a 1.5% agarose gel to ensure that single species of the amplicon were successfully amplified.

3.4.6 Formation of DNA Heteroduplex

Spin columns (e.g., QIAquick columns from Qiagen) were used to purify the PCR products according to the manufacturer's instructions. We typically eluted the purified amplicons in 50 μ l of 10 mM Tris-HCl (pH 8.5). To reanneal the amplicons, 200 ng of purified PCR product was diluted into 1 $\times$ NEB buffer 2 to a total volume of 20 μ l. The following steps were done to re-anneal the PCR product in a thermocycler: 95°C for 5 min, 95–85°C at -2°C/s, 85–25°C at -0.1°C/s, and hold at 16°C.

After denaturing and annealing, two types of homoduplex DNA and two types of heteroduplex DNA were formed. Since heteroduplex DNA migrates slower due to the formation of an open region between matched and unmatched genomic regions, homoduplex DNA and heteroduplex DNA were easily identified based on their mobility rate [56]. A representative heteroduplex formation is shown in Figure 5.

Figure 5: Formation of heteroduplex and homoduplex in the pcr annealing provided by wild type and mutant animals. The heteroduplex had a different mobility rate and was easily identified using polyacrylamide gel electrophoresis. The re-annealed DNA was used for polyacrylamide gel assays as described below.

3.4.7 Polyacrylamide Gel Electrophoresis (PAGE)

The reannealed 200 ng product was resolved using 10% polyacrylamide gel electrophoresis (PAGE; acrylamide:bis-acrylamide (29:1)) that was run at 120 V for 2–3 h. The gel was stained in 1× TBE buffer containing 0.5 µg/ml of ethidium bromide for 15 min before imaging. The presence of slow-migrating bands was indicative of DNA heteroduplexes. The amount of heteroduplex and homoduplexes was quantified using densitometry.

3.4.8 Mutagenesis Efficiency Calculation

To measure mutagenesis efficiency, we used the software Image lab 5.2.1 (Bio-Rad). Using this software, we estimated the heteroduplex bands intensity, compared to wild type bands. The mutagenesis rate was calculated as the percentage of heteroduplex DNA in the lane.

3.4.9 Analysis of F1 Animals

3.4.9.1 Sequencing

Samples were sent for sequencing. Prior to sequencing, it was possible to know where the InDel mutation occurred in the targeted genes of the founder fish.

3.4.9.2 Genotyping

In addition to PAGE analysis, we also used restriction digestion, using the enzyme HpyCH4IV, to identify homozygotes and heterozygotes, since it only cuts the wild type strand, but not mutant alleles of *Nr2e1*. The enzyme restriction location can be seen in Figure 6.

Figure 6: Restriction enzyme cuts *nr2e1* sequence in wild type animal. The expected bands following the restriction digestion are two bands for the wild type, one band for homozygotes, and three bands for heterozygotes.

For *prop1* genotyping, we used the BseRI enzyme, which cuts the homozygotes but does not cut wild type strand. The enzyme restriction location can be seen in Figure 7.

Figure 7: Restriction enzyme cuts *prop1* sequence in homozygous animal. The expected bands following the restriction digestion are two bands for homozygotes, one band for wild type, and three bands for heterozygotes.

Figure 8 includes a flowchart summarizing the CRISPR/Cas9 system.

Figure 8: Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) analysis summary. After the single chimeric guide (sgRNA) and Cas9 injection, we genotyped and found the fish which had the best mutation in the gene of interest.

3.5 Reproduction Rate

Two adult couples of zebrafish were selected for each genotype (i.e., wild type and the *nr2e1* mutant). They were added to the same tank the day before. A day after they mated, the eggs were counted immediately and kept at 28 °C. The numbers of eggs that survived were counted at 2, 8, 24, 48, and 72 h post-fertilization. The survival rate was expressed as a percentage. The total number of eggs per experiment related to the survival embryos at 72 h post-fertilization. These experiments were done three times on different days.

3.6 Immunofluorescence

Immunofluorescence on the wild type and *Nr2e1* homozygous zebrafish brain was performed at 10 days and 45 days post-fertilization, and in the adult fish. The antibody that were used were Nr2e1 anti-rabbit (Rb pAb to Nr2e1/tailless, ab30942, Abcam®), which was diluted at 1:50, and Sox2 anti-mouse (Anti-Sox2 antibody ab79351, Abcam®), which was diluted at 1:50. Anti-rabbit AlexaFluor® 488 dye (green; ThermoFisher Scientific), which was diluted at 1:200 was used to detect immunoreactions for Nr2e1, while anti-mouse AlexaFluor 633 (red; ThermoFisher Scientific), which was diluted at 1:200, was used to detect immunoreaction of Sox2. Further, DAPI (blue), which was diluted at 1:1,000, was used to detect cell nuclei. The Nr2e1 epitope was located before the protein truncation.

Immunofluorescence to detect hormones was also performed on the zebrafish brain at 10 days post-fertilization and in adulthood. Anti-GH anti-rabbit (Dako®), diluted at 1:50, was detected using the anti-rabbit AlexaFluor® 488 dye (ThermoFisher Scientific). Further, anti-guinea pig anti-TSH beta and anti-rabbit anti-FSH beta from the National Hormone and Peptide Program were also used, which were kindly donated by Dr. Sally Camper from the University of Michigan, MI, USA. All hormone antibodies, except anti-GH as noted previously, were diluted at 1:100 and the secondary antibody anti-rabbit AlexaFluor® 488 dye was diluted at 1:200, while anti-guinea pig AlexaFluor® 633 dye (ThermoFisher Scientific) was diluted at 1:200. Propidium iodide, which was diluted at 1:1,000, was used to stain nuclei in the zebrafish at 10 days post-fertilization.

3.6.1 Imaging

Images were acquired using a ZEISS LSM 510 Meta/UV and inverted, motorized, Microscope Zeiss Axiovert 200.

3.7 Behavior Test

We have developed an exploratory behavior assay to determine whether the *Nr2e1* mutation altered exploratory behavior in larval zebrafish, and if it showed a similar behavior phenotype to the mutant mice [33] and other species [57, 58].

This test consisted of measuring how many times the *nr2e1* homozygous animal, at the larval stage, explored the environment, compared to the wild type and the heterozygous zebrafish. Since zebrafish are usually non-aggressive and non-territorial, aggressiveness can be correlated with fearlessness.

We separated the three different lineages, i.e., homozygotes, wild type, and heterozygotes. For this test, a petri dish was filled with agarose and then cut to create a central chamber and surrounding ‘traps’. The entire agarose plate was submerged in a very shallow layer of zebrafish media. Larvae at 6 days post-fertilization from the same spawning were fed 5% egg yolk in 0.3× Danieau buffer for 2 h (Figure 9). They were cleaned and left for half hour in zebrafish media.

Figure 9: Zebrafish larvae feeding before the behavior experiment. Petri dishes with 60 larvae, who were separated based on genotyping, were fed with egg yolk 2 h before the experiment.

Zebrafish (6 days post-fertilization) were placed in the central chamber (Figure 10) and those that crossed the agarose barrier, which were considered the most exploratory, were collected after 10 min (Figure 11). The count of each genotype is shown, which was repeated three times on different days with different larvae to replicate the experiment. A total of 180 larvae (i.e., 60 wild type, 60 homozygotes, and 60 heterozygotes) were used for each experiment.

Figure 10: Petri dish representation for behavior tests. At 6 days post-fertilization, 60 larvae from each genotype, were used for the test, i.e., a total of 180 larvae were used per test. The larvae that crossed the agarose barrier were considered the most exploratory and were collected after 10 min.

Figure 11: Photograph of the exploratory behavior experiment. The larvae were able to go back and forward from the central square, but were collected when they are out of the main square for 10 min.

3.8 Statistical Analysis

A chi-square test was performed to check the significance of the results. Data were analyzed using the Sigma Stat 3.5 software, (San Jose, CA, USA). Statistical significance was set at $p < 0.05$.

4. RESULTS

4.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

4.1.1 Standardization of Gene Expression of *efa1*, *prop1*, and *nr2e1*

A single expected band was seen at 247, 375, and 1481 bp, for *efa1*, *prop1*, and *nr2e1*, respectively, from the wild type zebrafish analyzed at different time points. The gene expression of *prop1* and *nr2e1* were standardized to *efa1* (i.e., the endogenous gene) [49]

Figure 12: PCR amplified products of zebrafish cDNAs at different time points post-fertilization. amplification with primer pair a) *efa1*, b) *prop1*, and c) *nr2e1*. mm, molecular marker (1 kb DNA ladder); CN, negative control reaction.

4.1.2 *ccnd2a*, *p21*, *sox2*, and *efa1*

PCR was standardized for *ccnd2a*, *p21*, *sox2*, and *efa1* genes, using the single expected band at 216, 248, 197, and 247 bp, respectively.

Figure 13: PCR amplified products of the zebrafish cDNAs at different time points for *ccnd2a*, *p21*, *sox2*, and *efa1*. Amplification with the primer pair for A) *ccnd2a*, B) *p21*, C) *sox2* and D) *efa1*. MM, molecular marker (1 Kb DNA ladder); CN, negative reaction control; Hpf, hours post-fertilization.

4.2 Single guide (sgRNA) Generation for Gene Knockdown

Five sgRNAs were produced according to the protocol that was previously described (Item 4.3), namely two sgRNAs that specifically targeted and silenced a well-conserved region of *nr2e1*, and three sgRNAs that targeted the homeodomain coding region of *prop1*. The sgRNAs were quantified using the nanodrop (Thermo Scientific) (Table 6) and agarose gel (Figure 14). The result of quantification matched the bands seen in the gel, with Prop1-T2 yielding the highest score and strongest band.

Table 6: Nanodrop results for sgRNA generation

Gene	ng/μl
Prop1-1	24,8
Prop1-2	194,3
Prop1-3	180,1
Nr2e1-1	48,8
Nr2e1-2	107,7

Figure 14: Different samples of the five single guiding RNA (SgRNA). A 1.5% agarose gel was used for validation.

4.3 Injections

The cell was directly injected when the embryo was at the one cell stage. Only three sgRNAs, i.e. *prop1-2*, *nr2e1-1*, and *nr2e1-2*, worked well. Zebrafish injected with these three sgRNAs were kept alive until sexual maturation for genotyping and reproduction.

In total, we had the following:

prop1-2 = 40 fish

nr2e1-1 = 65 fish

nr2e1-2 = 165 fish

4.4 Knock-in Template Injection

The *prop1* knock-in template was injected using the *prop1-2* sgRNA since it showed better mutagenesis efficiency. If the template was properly incorporated, this would have led to two different bands in the gel, given that the restriction enzyme would have been able to cut the PCR product. However, since these two bands were absent from the gel, this meant that the template was not incorporated into the animal after injection (Figure 15).

Figure 15: PCR and XhoI digestion of the *prop1* injected zebrafish. a) The 1.5% agarose gel PCR product of the *prop1* reaction; 1–3, different individuals injected with *prop1*. b) XhoI enzyme digestion showed no point mutation; 1–3, different individuals injected with *prop1*. WT, wild type.

4.5 Mutagenesis Efficiency

Three pools of four embryos were chosen for this assay, genomic DNA was isolated, and then the target region was amplified using PCR and visualized using a 1.5% agarose gel (Figure 16).

Figure 16: Polymerase chain reaction (PCR) of different pools of injected embryos. The results from zebrafish injected with a) *nr2e1-1* and b) *prop1-2*

After the injection, the target region was amplified using PCR and analyzed with a heteroduplex mobility assay using PAGE (Figure 17).

Figure 17: 10% Polyacrylamide gel for genotyping. Results of the *nr2e1-1* and *prop1-2* samples. MM, molecular marker.

Among the five sgRNA tested only two worked well.

4.6 Mutagenesis Efficiency Calculation

Mutagenesis calculations were performed using Image Lab 5.2.1 Biorad®. The calculation consisted of analyzing the intensity of the mutant band compared to the wild type band. Based on the results of the polyacrylamide gel and mutagenesis efficiency calculation, we decided to breed three different clusters of injected animals as outlined below:

$$prop1-2 = 42\%$$

$$nr2e1-1 = 62\%$$

$$nr2e1-2 = 50\%$$

After they reached adult stage, we crossed them with the wild type (AB), for genotyping, sequencing, and analyzing the mutagenesis efficiency. The mutagenesis rates of *nr2e1* are shown in Table 7.

Table 7: Mutagenesis calculation based in the software Image Lab 5.2.1 Biorad®.

Sample number	Percentage of mutagenesis
18	44%
26	40%
34	55%
38	50%
39	70%

4.7 Genotyping

For genotyping, F0 fish were separated in tanks with wild types (AB) the night before copulation. The eggs were collected the following and raised to 24 h post-fertilization. The adults that did not lay eggs were returned to the normal tanks, while the ones that laid eggs were separated in cups with numbers until the genotyping results of the F1.

The eggs were collected in two pools of eight, and DNA extraction, PCR, heteroduplex formation, and PAGE were performed (Figure 18).

Figure 18: Genotyping of *nr2e1* males. Genotyping was performed using a 10% polyacrylamide gel.

After analyzing which pools harbored a mutation, single samples were analyzed and the ones carrying the mutation were sent for sequencing.

4.8 Sequencing

The individual F1 samples that showed mutation, when analyzed with PAGE, were sent for sequencing using Genewiz®. Some sequencing was also performed using LIM 42 by Illuminaseq. InDel mutations in the sample were determined using sanger sequencing. The F0 fish that yielded the highest percentages of frame-shift germline mutations were chosen for reproduction. We selected five fish for each gene. The selected fish are represented in Table 8.

Table 8: Samples chosen for reproduction, genes *nr2e1* and *prop1*

Females				Males			
Nr2e1 Sample	indels	Prop1 Sample	indels	Nr2e1 Sample	indels	Prop1 Sample	indels
18	10bp D	4	9bp D	1	10bp D	12	25bp D/I
34	10bp D	7	9bp D	11	5bp D	10	9bp D
39	10bp D					1	9bp D

D=Deletion; D/I=Deletion/Insertion

4.9 Chosen Mutant Lineage for *nr2e1*

Using the CRISPR/Cas9 system, we decided to use animals with a 10 bp deletion (ACGTCTATCC) in exon 4, which led to a frameshift mutation (Figure 19) in the *nr2e1* gene.

Figure 19: The *nr2e1* mutation diagram. Figure showing the 10 bp deletion in exon 4 (top) and the truncated protein (bottom).

All the F0 animals were sequenced. From F1 to the following offspring they were genotyped using a restriction enzyme after the PCR amplification, making it possible to determine homozygous, heterozygous, and wild type zebrafish.

The enzyme HpyCH4IV cuts the wild type, yielding two bands of 241 and 127 bp when analyzed using PAGE. However, only one band that is 368 bp is present in the *Nr2e1* mutant as there is no restriction site. Thus, the expected patterns of bands when visualized using PAGE is one band for homozygotes, three bands for heterozygotes, and two bands for wild type.

Figure 20: A 1.5% agarose gel with HpyCH4IV digestion illustrating the PCR product of *nr2e1*. HM, homozygous; HT, heterozygous; WT-wild type; MM, molecular marker.

4.10 Reproduction Rate is not altered

The survival percentage rate did not significantly differ between the three experiments ($p > 0.05$ and $\chi^2=0.724$). There was no difference in survival rate between the wild type and *Nr2e1* homozygotes (Table 9).

Table 9: Percentage of survival rate until 72 hours post fertilization

	Wild type	<i>nr2e1</i> homozygous
Experiment 1	66%	68%
Experiment 2	81%	51%
Experiment 3	66%	81%

The morphology of the *nr2e1* mutant zebrafish appeared normal from 2–72 h post-fertilization as shown in Figure 21.

Figure 21: Figure showing development of mutant and wild type zebrafish. No morphological differences were seen between the wild type and *Nr2e1* mutant.

4.11 Mutant Lineage for *prop1*

For *prop1* we found a 32 bp insertion at position 367

(AGGATGAAGAGGAGTACTACTACTTGGCTT) in exon 2, which led to a frameshift mutation (Figure 22) in the *prop1* gene.

Figure 22: Mutation diagram of *prop1*. A 32 bp insertion in exon 2 (top) and the resulting truncated protein (bottom).

4.12 Immunofluorescence

4.12.1 The presence of Nr2e1 in the Pituitary Gland

Nr2e1 is a known stem cell marker in the brain, which was also observed in the pituitary gland at different time points of a zebrafish life, as illustrated by the immunofluorescence imaging results that were acquired with confocal microscopy. In Figure 23, immunofluorescence of NR2E1 and SOX2 are shown in green and red, respectively.

Figure 23: Immunofluorescence in the wild type zebrafish brain for *Nr2e1* and *Sox2* at 10 and 45 days post-fertilization and during adulthood. The presence of *Nr2e1* in the pituitary gland and in the brain. Whole brain images were acquired at 10 days post-fertilization, showing the hypothalamus and pituitary region.

4.12.2 Immunofluorescence of *Sox2* and *Nr2e1*

The expression pattern of *Sox2* was elevated in homozygous animals (Figure 24). The epitope from *Nr2e1* in the antibody is expressed before exon 4, which is why it is present in the mutant.

Figure 24: Immunofluorescence of Sox2 and Nr2e1. The zebrafish brain of the wild type and homozygous mutant at 10 and 45 days post-fertilization, and during adulthood. Sox2 (1:50; red) was increased in the mutant compared to the wild type. Images were acquired using a confocal microscope at magnifications of 10× and 20×.

4.12.3 Immunofluorescence of Hormones

We tested some antibodies against some hormones, as illustrated in Figure 25, at 10 days post-fertilization to investigate differences in hormone expression between wild type and *nr2e1* mutant zebrafish.

Figure 25: Expression of Hormones at 10 days post-fertilization. The expression of growth hormone (Gh), follicle stimulating hormone β (Fsh β), and thyroid stimulating hormone β (Tsh β) was similar in the wild type and the *Nr2e1* homozygous mutant (acquired using confocal microscopy at 20 $\times$ magnification).

4.13 Exploratory Behavior

Homozygous mutant zebrafish were twice as more exploratory than the wild type. Exploration of the heterozygous zebrafish was not significantly different from wild type (Figure 26).

Figure 26: Comparison of Exploration by Zebrafish Larvae. This graphic demonstrates that homozygous mutant zebrafish were twice as more like to explore, compared to wild type and heterozygous. $*p \leq 0.05$

The mutant animal had a different behavior phenotype when compared to the wild type and the heterozygous, even at early stages of development.

4.14 Statistical Analysis

Statistical analysis was performed using a chi-square test to compare homozygous mutant and wild type values. Statistical significance was set at $P \leq 0.05$.

5. DISCUSSION

The present project aimed to characterize Nr2e1 in the pituitary gland, since both Nr2e1 and Sox2 are overexpressed in the *Prop1* mutant mice, namely the Ames mice that have multiple hormone deficiency.

Sox2 is a well-known marker of pituitary stem cells [14, 27, 59]. *Nr2e1* is a nuclear orphan receptor responsible for neurogenesis and maintenance of the brain, but its role in the pituitary had not been described before. Mice with an Nr2e1 mutation, named ‘Fierce’ mice, are aggressive, are difficult to mate, and lack maternal instincts, which make them challenging to work with [33]. Thus, zebrafish are a more feasible alternative animal model to characterize the role that *Nr2e1* plays in the pituitary gland. Thus, we generated a *nr2e1* mutant zebrafish model using the CRISPR/Cas9 system [51].

The *prop1* mutant zebrafish strain was difficult to establish in our laboratory, probably due to multiple hormone deficiency, which is a known cause for increasing the mortality rate in mice [60]. We still have to characterize *prop1* mutants that are generated using the CRISPR/Cas system to understand the role of Nr2e1 and its interaction with Prop1. To date, the only *prop1* zebrafish mutant previously reported in the literature, was created using the morpholino technique [61]. Since morpholino was not suitable to analyze the phenotype during adult life, given that it is diluted during development [62], we have no other zebrafish animal model to compare the survival rate.

The *nr2e1* heterozygous and homozygous zebrafish developed well and reproduced normally, once the homozygous survival rate did not significantly differ when compared to the wild type. Existent inter assay differences could be attributed to environmental causes, such as water quality, social environment, and handling [63].

Immunofluorescence of Sox2 was elevated in the brain of the *nr2e1* homozygous mutant at 10, 45 days post-fertilization, and during adulthood. A previous study using *Nr2e1* mutant mice, demonstrated that SOX2 positively regulates and dominantly controls the endogenous expression of *Nr2e1* [32, 64]. Conversely, NR2E1 exhibits negative feedback over itself [32]. Thus, we can hypothesize that the increased SOX2 expression in the brain is related to an imbalance in the circuit involving SOX2 and NR2E1, and its downstream genes.

FSH, TSH, and GH hormones are related to fertility rate and growth [65]. The expression of these hormones in the *nr2e1* mutant pituitary was similar to wild type. As *nr2e1* mutants presented a normal expected fertility rate, compared to the wild type, we can suggest that Nr2e1 is not crucial for the terminal pituitary differentiation, and hormone production and secretion. This is probably due to the overexpression of Sox2 when SOX2-positive cells evolve to terminal differentiation of somatotroph cells under injury [66].

NR2E1, which is also called TLX (orphan Nuclear Receptor tailless) in the human, has an important homology among different species. It plays an important role in maintaining social behavior. In drosophila and mice, the *Nr2e1* mutation has already been associated with behavior disorders. In humans it has been linked to neurological and psychiatry diseases such as aggression, schizophrenia, and bipolar disorders [57, 58, 67].

In the zebrafish larval stages aggressiveness is correlated with fearlessness. Based on this premise, our exploratory behavior assays were measured at 6 days post-fertilization. The *nr2e1* homozygous mutant showed a greater exploratory rate, compared to wild type and heterozygous zebrafish.

6. CONCLUSION

We generated two zebrafish lineages with a knockout to the *nr2e1* and *prop1* genes. We showed that *nr2e1* was important for the maintenance of social behavior, but was not required for hormone differentiation. Its presence in the pituitary gland probably has downstream effects on the stem cell marker, Sox2. However, its role in the pituitary remains to be determined.

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