Evaluation of Sex Differences in the Hippocampus and Pituitary of *Egr1* conditional knockout mice mediated by Nestin-Cre

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ABSTRACT

Early growth response 1 (*Egr1*) is a transcription factor critical for learning and memory in the hippocampus and pituitary cell differentiation. *Egr1* has been shown to extend continuation of the long-term potentiation in the hippocampus and is credited for forming long-term memories. The somatotrophs in the pituitary produce growth hormone and are found to be decreased in *Egr1*KO mice. These animals are also found to be sterile due to a decrease in LH_B, which blocks ovulation. All previous studies have evaluated these physiological processes with complete *Egr1*KO research strains or antisense oligonucleotides, up until now, no data specific to individual type of cells has been generated. In an attempt to focus on the understanding of the functions of *Egr1* gene in neural cell lineage, we are using an *Egr1*cKO Nestin-Cre model. Nestin allows for targeting neuronal lineage specific cells.

In **Chapter 1**, we provide a systemic view of *Egr1* gene and Nestin-Cre as a system for generating conditional knockout mouse strains. The Chapter begins with the identification of *Egr1* gene and its protein structure, then proceeds to grasp its link to memory with behavior testing. The critical role of *Egr1* in the pituitary and what cell populations are affected is also described. The same goes for Nestin-Cre, along with its limitations and understanding how to account for them in a study. The *Egr1*cKO Nestin-Cre system is the best form to understand neurological cell populations with *Egr1* removal.

In **Chapter 2** and **Chapter 3**, we employ the *Egr1*cKO Nestin-Cre mouse model to understand cell-specific knockout of *Egr1* in the nervous system by evaluating the hippocampus and pituitary. We explore learning and memory through behavioral tests and ribonucleic acid sequencing (RNA-seq) analysis to understand gene expression changes with *Egr1* removal. Females showed higher activity during behavior tests, with more movement in the elevated plus maze and lower freezing times during the contextual fear conditioning. RNA-seq had higher changes in females than males but was not affected by the Nestin-Cre system overall. The same RNA-seq changes in the pituitary gland were present, with females having higher genomic differentiation. Females had growth-specific pathways altered by Nestin-Cre.

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GENERAL AUDIENCE ABSTRACT

Genetics has become a very important forerunner in scientific research. One gene that has become important in many different research arenas is Early growth response 1 (*Egr1*). This particular gene is critical for learning, memory, and cell changes in the pituitary. In **Chapter 1**, we have analyzed the current research landscape of information on *Egr1* in its functions with learning and memory, as well as the pituitary. Most previous studies that have been completed only evaluate this gene by its removal from the entire body. This leaves a large gap in information about how this gene functions with specific cell types. To limit the type of cells from which *Egr1* has been removed, we have selected Nestin-Cre, a tool to remove genes from neuronal stem cells. The capabilities and limitations of this tool have also been explained in this chapter, along with how the two together can accomplish a cell-specific knockout of *Egr1*.

In **Chapter 2**, we have constructed an experiment with behavioral tests for mice, along with RNAseq data from the hippocampus to evaluate what changes have occurred in the *Egr1*cKO Nestin-Cre model. Female mice are more active in the behavioral test, including the elevated plus maze (EPM) and Contextual fear conditioning (CFC), than male mice. The same holds for differences in the RNAseq data as well.

In **Chapter 3**, the pituitary of *Egr1*cKO Nestin-Cre mice is the main focus. We evaluated RNAseq data and determined growth rates of transgenic mice. The mice had different growth rates over twelve weeks between the controls and the knockout. The RNAseq data also revealed many differences between males and females. Female mice had specific growth genes effects by the knockout of *Egr1*

DEDICATION

To my mother, nephews Leo and Luca, and in memory of Dr. Rebekah Brady

ATTRIBUTIONS

A number of colleagues assisted in design, data generation, and formation of Chapter 2 and Chapter 3 presented in this dissertation. Brief descriptions of contributions are below:

Hehuang Xie, Ph.D., is the Principal Investigator, assisted with design, interpretation, and writing of Chapter 2 and Chapter 3 presented here.

Yu Lin is a Ph.D. student in the Xie lab and assisted with formal data analysis in Chapter 2 and Chapter 3.

Yuze Zheng, is an undergraduate student in the Xie lab and assisted with formal data analysis in Chapter 2 and Chapter 3.

Xiguang Xu, Ph.D., is currently a research associate in the Xie lab. Dr. Xu generated immunohistochemistry in Chapter 2 and RNA scope in Chapter 3 as well as editing each chapter.

Min Liu, is the laboratory technician for the Xie lab and assisted with mice colony management, RNA isolation for Chapter 2 and Chapter 3.

Kurt Zimmerman, D.V.M., Ph.D., DACVP, is a collaborator with the Xie lab and provided immunohistopathology resources for Chapter 2 and Chapter 3.

Georgia E. Hodes, Ph.D., is a collaborator with the Xie lab and assisted with behavioral experimentation and data interpretation of Chapter 2.

Timothy Jarome Ph.D., is a collaborator with the Xie lab and assisted with behavioral experimentation and data interpretation of Chapter 2.

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Lastly, I want to acknowledge my perseverance, dedication, and fortitude. I have had several surgical procedures throughout this program, including brain and major abdominal. I have also had several weeks of management for chronic pain and health conditions that made progress stagnant and extremely challenging.

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List of Abbreviations

Egr1	Early growth response 1 (gene)
CA	Cornu Ammonis
CFC	Contextual fear conditioning
DEGs	Differential expressed genes
DG	Dentate Gyrus
DNA	Deoxyribonucleic acid
EC	Entorhinal cortex
EGR1	Early growth response 1 (protein)
*Egr1*cKO	Early gene response one conditional knock out
*Egr1*KO	Early gene response one knock out
EPM	Elevated Plus Maze
FSH	Follicle-stimulating hormone
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
HPA	Hypothalamic Pituitary Axis
LH	Luteinizing hormone
LTM	Long term memory
LTP	Long term potentiation
MAPK	Mitogen activated protein kinase
NMDA	N-Methyl-D-Aspartate
PCA	Principal component analysis
Pitx1	Pituitary homeobox factor 1
PRL	Prolactin
RNAseq	Ribonucleic acid sequencing
sf1	Steroidogenic factor 1
TET1	Ten eleven translocation protein 1
TSH	Thyroid-stimulating hormone

Chapter 1 Review of Egr1 and Nestin Hippocampal Learning and Memory and Pituitary Growth

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1.1 Abstract

Early growth response 1 (*Egr1*) is a transcription factor important for learning and memory and documented to extend continuation of the long-term potentiation in the hippocampus. It is critical for pituitary cell differentiation and needed for somatotropes that produce growth hormone. Nestin-Cre is a system used to eliminate targeted genes specifically in neuronal stem cells and has been beneficial for studying gene function in the nervous system. Nestin-Cre mice are reported to have smaller sizes than controls. These two factors, Nestin-Cre and the floxed *Egr1* alleles, are being reviewed in the following paragraphs to understand how each plays an important role in the physiological function of learning and memory and pituitary function for a study designed to evaluate the two processes.

Keywords: pituitary, learning and memory, Hippocampus, Egr1, growth, Nestin-Cre

1.2 Introduction

In 2021, mental health disorders affected 57.8 million adults in the United States, with 51.7% being women (*Mental Health by Numbers*, 2023). Alterations in memory formation are key to many mental health conditions, such as post-traumatic stress injury, anxiety, and dementia (Gallo et al., 2018). However, treatment options for these memory impairments remain limited. Understanding the molecular mechanisms of memory formation, such as *Egr1*, may lead to a way to help patients adapt and heal from poor mental health conditions that result from memories formed from traumatic events.

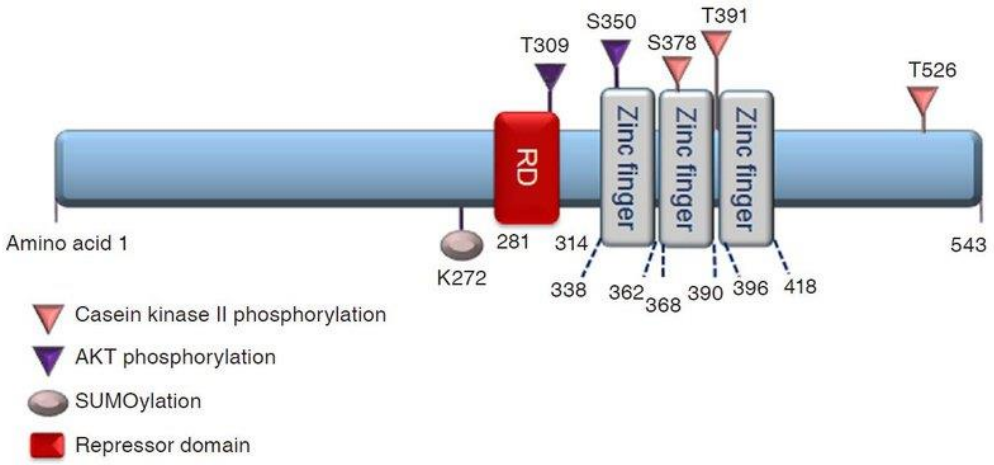
1.3 Early Growth Response 1

1.3.A History, Structure, and Pathways

Early growth response 1 (*Egr1*) is a transcription factor immediately upregulated by neuronal stimulation (Madabhushi et al., 2015). It goes by different aliases, such as Zif-268, NGFI-A, Krox 24, or ZENK, depending on the field of study or the name given by the lab that originally described its function. EGR1 was found in the 1980s after an experiment was conducted in rat PC12 cells looking for genes that immediately respond and upregulate (Bozon et al., 2002; Milbrandt, 1987). Early growth response 1 gene *(Egr1)* is from a larger family of genes

called Immediate early genes (IEG). This gene family encode transcription factors that do not require de novo protein synthesis to become activated. After immediate stimulation, mRNA increases within thirty minutes (Minatohara et al., 2015). *Egr1* is one of four different early growth response (Egr) members within the early growth response family. *Egr2* and *Egr4* are both associated with hindbrain development, and *Egr3* is associated with muscle, lymphocyte, and neurodevelopment. All genes in this family have a three-zinc finger sequence with a GC rich DNA binding domain (Figure 1.1) (Beckmann & Wilce, 1997). The zinc fingers have a binding sequence of GCG(G/T)GGGCG and are highly homologous among the early response gene family, suggesting that family members may bind to the same sites of a subset of the same target genes. In the structure, phosphorylation has been known to either enhance or restrict *Egr1* transcriptional activity. The repressor domain also inhibits Egr1 transcriptional activity (Gallo et al., 2018).

Figure 1.1: EGR1 Structure



Note: From Magee, Nancy & Zhang, Yuxia. (2017). Role of early growth response 1 in liver metabolism and liver cancer. Hepatoma Research. 3. 268. 10.20517/2394-5079.2017.36.

There are several different signaling pathways in which Egr1 can be activated in a cell. This activation can be led by a major category pathway called mitogen-activated protein kinases (MAPK). MAPK pathways are signaling cascades initiating from a receptor tyrosine kinase at the plasma membrane and concluding in a terminal kinase, for which the pathways are named. The c-JUN N-terminal kinase 1, 2, and 3 (JNK1/2/3) pathway is activated during stress such as trauma, neoplasia, and inflammation, the extracellular regulated kinase 1 and 2 (ERK1/2) is stimulated by growth factors, and the p38 MAPK pathways from lipopolysaccharide exposure.

The p38 MAPK pathways can also be stimulated outside the cell by Ca^{2+} influx from pathogen exposure (Banerji & Saroj, 2021).

N-methyl-D-aspartate receptors are the most common receptors associated with *Egr1* stimulation in the body. After the rodent striatum was stimulated with quinolinic acid, an NMDA agonist, several IEGs, including *Egrl,* were upregulated (Shan et al., 1997). When approaching the expression of EGR1 a different way, using a competitive antagonist of NMDA, such as 2-amino-5-phosphonopentanoic acid, after a noxious stimulus to the spinal cord, EGR1 was reduced (Rahman et al., 2002). Considering the induction pathways and downstream targets that Egr1 regulates is important.

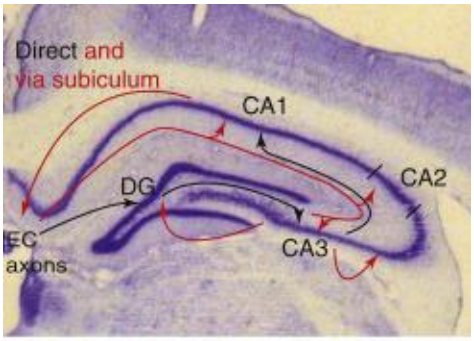
EGR1 can recruit the Ten-Eleven Translocation protein 1 (TET1) after binding to methylated DNA and removing the methylated epigenetic marker to activate downstream targets. This recently discovered phenome allowed us to understand how *Egr1* can interact with epigenetic marks on DNA and allow transcription to occur. (Sun et al., 2019). A few of those target categories include factors in apoptosis, tumor suppressors, and immune responses. In the early immune response, the cytokine tumor necrosis factor binds to *Egr1* through its GC right promoter region and then influences a proinflammatory state. (Woodson & Kehn-Hall, 2022).

1.3.B Memory and Hippocampus

EGR1 has been implicated in the hippocampus as a critical regulator of long-term memory formation (Davis et al., 2010; Yagi et al., 2016). Memory in its most basic form is the brain's ability to take information that it has recently acquired, store it, and use it later upon recall (Tonegawa et al., 2015). Memory can be classified into active and inactive memory. Active memory includes short-term, immediate, and working memory, while inactive memory comprises long-term, reference, and passive memory (Miller, 2021). The hippocampus is an important brain structure required for memory formation and has been associated with long-term memory even as far back as the 1950s. Patients like Henry Molasion, known as H.M., had a larger portion of his hippocampus removed, leading to profound memory deficits (Scoville & Milner, 1957).

The hippocampus is a structure in the brain that is important for learning and memory. The structure is placed in the brain's medial temporal lobe beneath the cortex. It is important to distinguish the hippocampus from the cortex outer layer of the brain (Knierim, 2015). It can be broken down into four anatomical parts: the Cornu ammonis (CA), dentate gyrus (DG), subiculum, and entorhinal area (EC) (Figure 1.2). These main parts are broken down even further into specific regions, which has led to an understanding of how plastic changes take place in the hippocampus and what region is responsible for different aspects of studying memory in the hippocampus. The pathway for information to start the learning process begins in the EC and has the strongest connection for input into the DG. From the DG, signals make their way around the CA, broken down into CA4, CA3, CA2, and CA1. From the patient, Henry Molasion and the discovery of long-term potentiation (LTP), which was discovered in the hippocampus, has established this structure as critical for memory and remains the target structure of many memory-based studies in the brain. (Anand & Dhikav, 2012).

Figure 1.2: Coronal slice through the transverse axis of the hippocampus



Note: From James J. Knierim,The hippocampus Current Biology,Volume 25, Issue 23,2015, Pages R1116-R1121,ISSN 0960 9822, https:/ / doi.org/ 10.1016/j.cub.2015.10.049.(https://www.sciencedirect.com/science/article/pii/S0960982215013123)

LTP is a rapid and long-lasting increase in the strength of synapses. It was officially demonstrated in rabbits during anesthesia in the DG of the hippocampus (Nicoll, 2017). The amplitude of signals from the cells in the DG consistently increased with tetanic stimulation, as well as decreased latency to reaction time. The stimulations produced plasticity of neurons from 30 minutes up to 10 consecutive hours in the brain (Bliss & Gardner-Medwin, 1973). LTP can be broken down into early and late phases. A single tetanus can produce the early phase of the LTP, which can last from one to three hours. Several stronger and repetitive tetani can produce the late phase, which can last up to twenty-four hours, and this phase requires transcription factor activation and protein synthesis for maintenance. (Baltaci et al., 2019).

The late phase of LTP is where EGR1 has been documented to be critical for memory development. NMDA receptor activation in the hippocampus, from high frequency, causes an immediate and strong upregulation. *Egr1* is most correlated to maintaining LTP and induces

upregulation in all LTP stimulating situations (Abraham et al., 1991; Cole et al., 1989). The immediate upregulation of *Egr1* is also seen in the spinal cord with noxious stimulus. The dorsal horn in the spinal column follows closely to the function of *Egr1* in the hippocampus with upregulation of *Egr1* affecting the long-term maintenance inflammatory hyperalgesia (Rygh et al., 2006; Wisden et al., 1990), giving support for *Egr1* needed for long term plastic changes. When *Egr1* is absent, such as in knockout animal models, the late phase of LTP is not maintained, unlike wild type controls, where normal *Egr1* is present in the body. The later phase of LTP has a significant spike potentiation during the phase that is maintained in wild type controls from 24 to 48 hours, where in contrast, the knockout and partial knockout animals are not able to maintain spike potentiation at either of the same times. These changes from a lack of late phase LTP in the hippocampus are demonstrated abnormalities in long term memory behavioral testing assays, while short term memory remained intact (Jones et al., 2001).

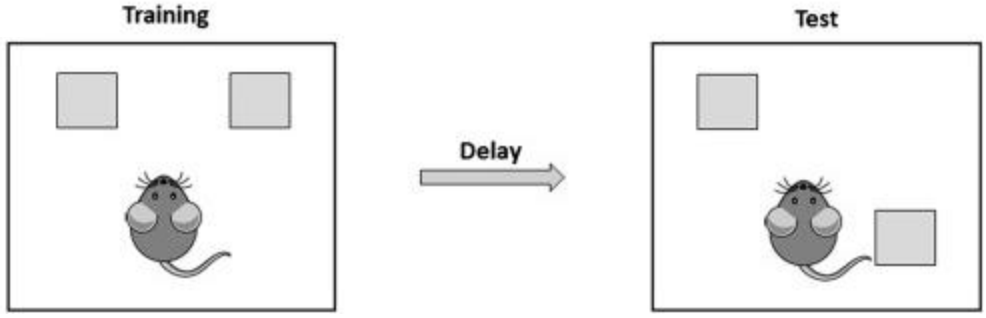
1.3.C Behavior Testing

Animal behavior testing enables a more robust understanding of how a specific gene change can affect the phenotypic response of an organism. These changes, especially in rodent models, are often used to study neuropsychiatric diseases. Although no an animal is perfect to model human diseases, animal models do give the research community a stable foot with inception to understand possible molecular targets ("Building a better mouse test," 2011). Several behavioral assays have been used in models to study *Egr1* and further expand on knowledge of its role and capabilities in the body.

Novel spatial object testing is one memory test that has provided evidence of a significant difference in the learning and memory of knockout *Egr1* mice. This particular test is very useful for evaluating hippocampal function since the hippocampus is the location of spatial learning cells in the CA1 (Renaudineau et al., 2009). A mouse will undergo a training period where it is exposed to an arena with similar objects placed strategically (Figure 1.3). The mouse is allotted a time frame to explore objects within the arena over specific days. The training period allows the mouse appropriate exposure time to recognize objects in specific locations. Once the mouse has completed an appropriately planned training protocol, one of the objects is moved to a different location within the arena. This object is considered a novel spatial object since it has been

moved. The testing day follows immediately, twenty-four hours after the final training day. The mouse is placed in the same arena with the new layout since one object has been moved, and the remaining familiar object is in the same location as during training. Time is recorded for the exploration of either of the objects in the arena. If hippocampal learning has taken place, the mouse will remember spatially the object in the same place and explore the novel object that has been moved specifically for testing (Denninger et al., 2018). *Egr1* knockout mice have displayed longer or the same amount of exploration time with the familiar object than control subjects exploring the spatial novel object in the same testing assays (Jones et al., 2001; Penke et al., 2014).

Figure 1.3: novel object location



Note: Adapted From Rose Chesworth, Georgia Watt, Tim Karl,Chapter 31 - Cannabinoid Modulation of Object Recognition and Location Memory—A Preclinical Assessment,Editor(s): Abdel Ennaceur, Maria Angelica de Souza Silva,Handbook of Behavioral Neuroscience,Elsevier,Volume 27,2018,Pages 461-488,ISSN 1569-7339,ISBN 9780128120125.

Egr1 knockout mice have also been subjected to contextual fear conditioning testing and shown many differences compared to control mice, suggesting that *Egr1* does have a specific role in fear memory. Contextual fear conditioning is where an animal is trained to associate an adverse stimulus, such as an electric shock, without a specific condition. This form of fear conditioning is often used to study fear memory and learning, which can be related to many different psychiatric disorders. This type of behavioral testing works with two different neural pathways: the hippocampus and the amygdala (Savage & Ma, 2014). A mouse in training for this conditioning spends approximately a week in training prior to exposure to the testing chamber. Training includes three days of handling for five minutes daily in the mouse's home cage. This allows the animal to acclimate to touch from the handler and decreases potential anxiety related to handling during training. The remaining four training days involve transporting the animal in its cage to the room where the experiment will take place. After the week of training is complete, the mouse is placed into a chamber with a wire grid on which it can receive a shock. After the

shock experiment, the mouse is immediately placed back in its home cage. The following day, the mouse is placed into the chamber again with no shock administered and monitored for freezing behavior which is evaluating specifically hippocampal learning behavior. This freezing behavior represents what the mouse learned during the testing day and if the chamber is the context to the foot shock, recalling a fear memory (Schöner et al., 2017). This is one of many different protocols to approach fear conditioning with foot shock and can be altered depending on what stage of learning and memory a particular researcher might be interested in exploring.

In mice with complete *Egr1* knockout, there are reported deficits in consolidation and reconsolidation during contextual fear conditioning with foot shock. Mice that were heterozygous genotype for *Egr1* only having partial knock down demonstrated deficits in reconsolidation only and suggested a gene dosage effect during learning and memory (Besnard et al., 2013). After contextual fear conditioning, it has also been found that EGR1 is increased in the dorsal hippocampus for approximately thirty minutes and peaks one hour after the initial testing (Lonergan et al., 2010). The hippocampus CA1 neurons show *Egr1* upregulation during retrieval of contextual fear conditioning and not cued. The upregulation was limited to recent memories less than twenty-four hours old and showed no *Egr1* upregulation with memories older than twenty-eight hours (Hall et al., 2001).

The studies presented have been produced from test strains of animals that are universal knockouts for *Egr1* or from antisense oligonucleotides to a brain region targeted with learning and memory. However, this process still lacks region or cell type specificity (Malkani et al., 2004). The current literature concerning *Egr1* and its cell-specific role in the brain during learning and memory is insufficient. The proposed work will use a conditional knockout mouse model. This model will target *Egr1* expression from primarily neuronal lineage cells in the central nervous system. This will allow for targeted cellular data and limit the knockout expression from all body cells, like previous universal knockout models, to only those targeted in the central nervous system. Future studies should be conducted to limit the regions of interest in the brain and cells in the central nervous system that express *Egr1* to better understand the machinery of this important transcription factor.

1.3.D Pituitary

Egr1 was an early target for important pituitary function when *Egr1* knockout mouse strains were developed. As the animal strains were produced, it was noted that knockout females did not reproduce offspring, and all knockout animals were smaller in size compared to wild type controls (Topilko et al., 1998). This led to further investigations of the pivotal roles that *Egr1* takes on in the body.

The pituitary gland is crucial for many biological processes, including reproduction, growth, metabolism, and overall homeostasis. This gland is located underneath the brain in the skull and is positioned in the Sella turcica, which is a depression in the sphenoid bone. The pituitary is connected to the brain through the median eminence of the hypothalamus via the hypophysial stalk, also known as the infundibulum, which has both a neuronal and a vascular component. Anatomically, the gland comprises three distinct lobes: the anterior, intermediate, and posterior. Embryonically, the anterior and intermediate lobes arise from the oral ectoderm, which invaginates to Rathke's pouch and forms the lobes. The posterior lobe originates from the neural ectoderm. Each lobe has specific cell populations and makes hormones for different body areas to function in prime performance (Anastassiadis et al., 2019).

Each lobe has a distinct function and cell types present; such as, the intermediate lobe has melanocytes, and the posterior lobe produces oxytocin and vasopressin (Alatzoglou et al., 2020), none of which have been linked to *Egr1*. The majority of this review will focus on the anterior lobe of the pituitary, which *Egr1* has been known to be pivotal. The anterior lobe of the pituitary has five distinct cell populations that produce different hormones: gonadotropes, somatotropes, thyrotropes, corticotropes, and lactotropes. Gonadotropes are a minority population in the anterior pituitary, with about 5-15% of cells. This population produces luteinizing hormone (LH), and follicle stimulating hormone (FSH), and works with the gonadal feedback system through the hypothalamus. These hormones are responsible for reproduction and stimulate the gonadal tissues in the male testes and female ovaries to have a functional and successful reproductive system (Clay et al., 2020). Somatotropes are one of the largest populations of cells in the pituitary. Somatotropes are responsible for producing growth hormone, which is responsible for growth, development, and metabolism. Males have a higher population of somatotropes in the anterior pituitary than females (Miles et al., 2020). Thyrotropes are one of the smallest populations in the anterior pituitary, with only 5% or less of total cells in the

pituitary. This type of cell produces thyroid stimulating hormone, works directly with the thyroid gland, and is a pivotal part of the hypothalamic thyroid axis (Cheung et al., 2023). Corticotropes are the first cells to terminally differentiate to hormone producing cells. This group is important in producing adrenocorticotropic hormone, also known as (ACTH). This particular hormone works in the hypothalamic pituitary axis (HPA) and is a very critical player in natural responses such as fight or flight. This hormone is often a key factor in stressful situations for the body (Drouin, 2022). The final cell type, lactotropes, have a larger representation in females over males and produces prolactin. Prolactin is critical for mammary development and enzymes for milk formation (Sadiq & Tadi, 2023).

1.3.E LHB and Growth Hormone

Female *Egr1* knockout mice were determined to be infertile early on in the development of animal knockout gene strains, as they were not able to produce offspring. The cause of infertility was determined to be a deficiency in luteinizing hormone beta chain (LH_B). Since it was unknown which part of the system the deficiency was coming from, these mice were ovariectomized. Without the ovaries producing a feedback loop to the pituitary, follicle stimulating hormone beta chain (FSH_B) increased but not LH_B, pointing directly to the pituitary (Lee et al., 1996). The loss of *Egr1* did not increase the expression of other *Egr* family members in the gonadotropes. Further research using an equine LH_B indicated two *Egr1* binding sites in the promoter region of LH_B, and the response to GnRH depends on the *Egr1* promoter binding (Wolfe & Call, 1999). When looking at the female rat estrus cycle specifically, *Egr1* has a drastic increase in mRNA presence and, respectfully, a decrease during diestrus (Gajewska et al., 2014). Males do not show the same infertility as females do when *Egr1* is knocked out. This is due to ERK-1, one of the regulating pathways of *Egr1*, not being required for male fertility like it is for females. The ERK pathway upregulation of *Egr1* is specifically needed for LH_B, which causes the sex difference in fertility when *Egr1* is knocked out. Males also have a selective population of gonadotropes that express *Egr1*, which helps maintain the association between *Egr1* and LH_B (Bliss et al., 2009; Man et al., 2014**).**

Even though the female has specific fertility complications due to *Egr1* removal, the male does show difference in growth patterns of body size like the female. When histopathology has

been completed on pituitary from animals of *Egr1* knockout condition, there is a decreased number of somatotropes present. Since somatotropes are responsible for GH production, the decreased number of these cells directly decreases GH in the body. This is seen in both males and females and has been tracked with a growth curve over a six-month period. Both males and females had a direct decline in growth starting at two weeks, separating them from controls that have *Egr1* present (Topilko et al., 1998).

1.4 Nestin

1.4.A History, Expression, and Pathways

Nestin, also known as neuroepithelial stem cell protein, is a cytoskeletal protein classified as an intermediate filament. It was specifically named Nestin because of its expression in neuroepithelial stem cells (Lendahl et al., 1990). Expression can be found in various types of cells, including the pancreas, small bowel, muscle, kidney, neural stem cells, testes, hair follicles, and the non-hematopoietic fraction of the bone marrow (Bernal & Arranz, 2018). There are six described intermediate filaments with Nestin specifically classified as type VI. It is very similar in structure to type III and type IV. However, due to an α-helical region and its unique short N-terminus end and long C-terminus end, it remains in its own class (Steinert et al., 1999).

Nestin has been implicated in proliferation of stem cells with asymmetric cell division. During mitosis, Nestin reorganization is attributed to phosphorylation that partially disassembles Nestin filament (Gómez-López et al., 2014; Sahlgren et al., 2001). Differentiation of cells is also linked to Nestin, with stem cells having a higher expression and lower expression with cells that have split and committed to a new cell state. In myoblast specifically, Nestin can inhibit cyclin-dependent kinase 5, by regulating cleavage of the activator protein p35 to its degradation-resistant form, p25, which stops the differentiation (Pallari et al., 2011). Migration is reported to be affected along with other cell properties by Nestin. This phenomenon is mostly linked to neoplastic cells, which is not completely understood now (Hyder et al., 2014).

1.4.B Nestin-Cre

Since Nestin has higher expression in neuronal stem cells, it is an excellent candidate to pair with the Cre-LoxP system to remove specific genes by deletion in the nervous system. Cre is the first part of the Cre-LoxP and is a site-specific recombinase technology that causes recombination of specific sequences of DNA**.** The Cre has a target site which is the LoxP, a specific 13bp that flanks a target gene for deletion from the DNA (Branda & Dymecki, 2004). Whenever Nestin-Cre is expressed in Nestin expressing cells, the Cre will then target any gene flanked with the LoxP sites to remove the gene from DNA.

Even though Nestin-Cre makes an excellent method for targeting gene deletions from the central nervous system, it does not come without biological influences. In an early study, Nestin-Cre was used to remove a glucocorticoid receptor in mice, and these particular animals had decreased anxiety responses in many different behavior tests (Tronche et al., 1999). A later study was designed to consider if the transgene Nestin-Cre was causing the behavior difference versus the actual knockout of the glucocorticoid receptor. Most emotional behavior tests for Nestin-Cre alone were reported to have normal outcomes and did not affect the behavior results from the test. However, Nestin-Cre only mice did have impaired acquisition of cued contextual fear memories (Giusti et al., 2014).

Nestin-Cre has also been attributed to mild hypopituitarism when it was noticed that mice with only Nestin-Cre had smaller sizes than controls. An early report published findings of high Nestin-Cre transgene activity in the adult pituitary (Gleiberman et al., 2008); however, several later studies were not able to reproduce the same findings. Due to poor recombination in the adult pituitary, hypopituitarism is not due to significant transgene activity in the pituitary of Nestin-Cre mice. Adult Nestin-Cre mice display a 70% to 80% reduction in GH, PRL, and TSH (Galichet et al., 2010). These findings still do not explain why Nestin-Cre has mild hypopituitarism and leaves a gap in scientific knowledge of this system.

1.5 Conclusion

Egr1 is an important transcription factor for learning, memory, and pituitary function. The current literature is insufficient for brain cell specific *Egr1* interactions and understanding the true effects on growth with Nestin-Cre. *Egr1* has been known to cause specific memory issues when completely removed from the body, such as LTM and spatial memory abnormalities. The Nestin-Cre system is a drastic way of conditional knockout to down-size the affected cellular populations to a focused group of cells. Nestin-Cre itself can cause potential anxiety-related learning differences and growth disturbances. Due to the previously reported changes caused by Nestin-Cre, like mild hypopituitarism and impaired cued fear conditioning, it is not only important that the official conditional knockout should have a control, but so should include the Nestin-Cre system itself, so any results can be appropriately interrupted. *Egr1* conditional knockout Nestin-Cre mediated mice will be an excellent model to study cell-specific Egr1 roles in memory, learning, and pituitary function.

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Chapter 2 Sex Linked Behavioral and Hippocampal Transcriptomic Changes in Mice with Cell-type Specific Egr1 Loss

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2.1 Abstract

The transcription factor EGR1 plays critical roles in various neurological processes, including the response to stress, learning, and memory. Egr1 complete knockout mice demonstrate decreased depressive or anxiety-like behavior and impaired performance in spatial learning and memory. In this study, we cataloged the behavioral and transcriptomic character of Nestin-Cre mediated *Egr1* conditional knockout (Egr1cKO) mice and their controls. Although the conditional knockout did not change nociceptive responses or anxiety levels, it did trigger females to have increased activity levels during testing. The hippocampal dependent process of spatial learning was not affected, but females did have a lower recall ability after training during the contextual fear learning than males. RNA-seq data analyses revealed that the sex-related differences were amplified in Nestin-Cre mediated *Egr1* conditional knockout mice. In addition, in males, the influence of Egr1cKO on pituitary gene expression may be overridden by the Nestin-Cre driver. Differential genes associated with Nestin-Cre driver were significantly enriched for genes related to growth factor activity and binding. Altogether, our results demonstrate that Nestin-Cre and the loss of *Egr1* in neuronal cell lineage have distinct impacts on hippocampal gene expression in a sex-specific manner.

Keywords: Sex difference, *Egr1*, Nestin, RNA-seq, hippocampus, behavior

2.2 Introduction

The early growth response factor 1 (*Egr1*) is an early response gene that can be rapidly and transiently induced in response to various environmental stimuli[1-3]. As a transcription factor, the EGR1 protein directly binds to genomic DNA containing a motif with CpG dinucleotides and serves as a critical epigenetic regulator for downstream gene activation[4]. Through the regulation of gene expression, *Egr1* plays an essential role in learning and memory processes[5]. Compared to wild-type mice, *Egr1* complete knockout mice showed deficits in long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus. The performance of *Egr1* complete knockout mice was impaired in various tests requiring long-term memory, including spatial and

object recognition memory[6, 7]. *Egr1* is also involved in the stress response and has been implicated in various stress-related disorders, including depression and anxiety. For instance, decreased depressive-like behavior was observed in rats with local infusion of antisense oligodeoxynucleotide to specifically knockdown *Egr1* in the dorsal hippocampus[8]. Additionally, the contextual fear conditioning (foot shock) test showed that the memory reconsolidation process involves the recruitment of *Egr1,* and the knockdown of *Egr1* interferes with learning and memory processes of fear without affecting freezing behavior[9, 10]. A previous study has investigated the effects of *Egr1* loss on hippocampal genome-wide gene expression using cDNA microarray[11]. A total of 368 differentially expressed genes were identified in the hippocampi from *Egr1* complete knockout mice exposed to different phases of a fear conditioning paradigm. Despite these previous studies performed with complete knockout mice or regional *Egr1* knockdown with antisense oligodeoxynucleotide, little is known about the changes in behavior or gene expression profile associated with the cell type-specific loss of *Egr1*.

Male and female mice are reported to have different behavioral responses to external stimuli[12]. The interaction between gonadal and stress hormones, specifically estrogens and glucocorticoids, is responsible for the differences in stress susceptibility between the sexes. Decades of studies have also broadened our comprehension of how EGR1 regulates hippocampal function, particularly in relation to sex differences. An early report has shown that the expression levels of *Egr1* and other immediate early genes in the hippocampus were differentially regulated during the formation of the intromission mnemonic, a learned association between sexual behavior and reward[13]. Normal aggressive behaviors and social recognition memory depend on the proper functioning of vasopressin 1b receptor (Avpr1b) in the CA2 region of the hippocampus. In Avpr1b knockout mice, the intruder-evoked *Egr1* expression was observed in males but not in female mice[14]. Sex differences in corticosterone receptors in the hippocampus have been reported[15]. Only in female mice with corticosteroid-binding globulin knockout was the mRNA expression of Egr1 reduced in the hippocampus after the stress induced by the force swim test[16]. However, the influence of *Egr1* loss on sex-linked behavior and hippocampal gene expression profile remains unexplored.

The Nestin-Cre transgenic mouse is one of the most frequently used mouse models to study gene functions in the central nervous system. In this study, we used the Nestin-Cre driver to obtain *Egr1* conditional knockout (Egr1cKO) mice with the neuronal-specific loss expression of the

Egr1 gene. We examined the behavior changes and evaluated the sex-related differential gene expression in the hippocampi of Egr1cKO mice along with Nestin-Cre controls.

2.3 Material and Methods

Animals. All animal experiments were performed according to the Institutional Animal Care and Use Committee guidelines at Virginia Tech (Blacksburg, VA, Animals. All animal experiments were performed according to the Institutional Animal Care and Use Committee guidelines at Virginia Tech (Blacksburg, VA, USA). The Egr1 conditional knockout mouse strain (Egr1_tm1a_A04, C57BL/6N-Egr1/Tcp; MGI:5766027) was purchased from the Centre for Phenogenomics, Canada. The Nestin-Cre (B6.Cg-Tg (Nes-Cre)1Kln/J; Jackson Lab, #003771) was a kind gift from Dr. Michael Fox's lab. To create the Egr1cKO mice, a breeding scheme was implemented to cross individuals with varying degrees of floxed Egr1 alleles with heterozygous Nestin-Cre mice until the desired Nestin-Cre driving Egr1cKO was achieved. The mice were maintained and bred in a 12-hour light/dark cycle under standard pathogen-free conditions. Teklad Global 18% Protein Rodent Diet was provided with free food and water access. For all behavioral experiments (n=10 per sex/group) we used male and female mice with four different genotypes ($Egr1^{f}Nes^{cre+}$, $Egr1^{f}Nes^{cre-}$, $Egr1^{wt}Nes^{cre+}$, and $Egr1^{wt}Nes^{cre-}$),

Genotyping PCR. Mice were genotyped at three weeks of age during the time of weaning. A small distal portion of the tail was removed and incubated at 550C overnight in DirectPCR (Tail) (VIAGEN, cat# 101-T) solution along with Proteinase K (ThermoFisher, AM2546). The next day the sample was incubated at 800C for one hour to deactivate the proteinase K before setting up PCR sampling. Genotyping PCR reactions were performed according to the Jackson Laboratory's protocol for Nestin-Cre and the Centre for Phenogenomics' protocol for *Egr1-loxp*, as described in our previous study.

Western blotting. Total cellular proteins were extracted from tissues using lysis buffer, separated in the 10% SDS-PAGE gel, and transferred to PVDF membranes. After blocking with 5% skimmed milk for 1h, and then the membrane was incubated with the primary antibodies at 4 ℃ overnight. The primary antibodies against EGR1 (CST, cat# 41542, 1:1000 dilution) and

GAPDH (CST, cat# 2118, 1:2000 dilution) were used. The next day, the membrane was incubated with specific secondary antibodies at 25 °C for 1 h. After three washes with PBST buffer, the membrane was incubated with Super Signal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher, cat# 34580) and visualized using the Bio Rad ChemiDoc imaging system. GAPDH was included as an internal control.

Immunohistochemistry (IHC) Egr1cKO mice and controls were rapidly and deeply anesthetized with isoflurane (Vet One, cat# 502017) and perfused transcardially with 10% formalin into the left ventricle. The right ventricle was opened to allow for exsanguination. After cardiac arrest was confirmed, the mice went through cervical dislocation. The brain was exposed with maximum bone removed to allow for further tissue fixation in 10% formalin overnight. Once fixed, tissues were sent to the Virginia-Maryland College of Veterinary Medicine pathology laboratory for embedment. Coronal slices were taken of the brain near the hippocampus in the wax block and provided back to the pathology laboratory. The coronal brain sections went through an automotive system with a Ventana Discovery Ultra machine (DAB Detection kit Cat#: 760-159) and secondary antibody, Omap anti-Rb HRP (Roche, Cat#:760-4311). The brain section was stained with anti-EGR1 rabbit antibody (CST, cat# 41542). Images were acquired using a MoticEasy Scan Pro 6 slide scanner.

General procedure for animal behavior tests

The testing cohorts were allowed 24 hours in between different behavioral assays before going on to the next test. Every experiment took place at 2:00 pm, for consistency. All animals were habituated to the testing room 30 minutes before each test. Most behavioral tests were performed in red light to not increase anxiety levels from the environment; normal lighting conditions were used for the two sensory tests with heat for protection of the administrator of the test and the animals. To minimize other factors, such as sex impeding on results of different cohorts, all male animals went through behavior testing before any female testing. All testing arenas or devices were cleaned with 75% alcohol before and after each test. Cohorts of male or female animals were tested in groups as genotypes became available through the breeding schematic designed for maximizing desired genotype outputs.

Open Field. Noldus information Technology Inc. behavioral system Ethos XT 17 was used to track behavior in the open field maze. Predefined Open field Maze arena from the Ethos XT 17 software was used as a template and then modified for specific parameters set for the open field maze. A square box approximately 50cm x 50cm x 50cm was used for the open field arena. The mouse was placed into the center of the box and allowed to explore for 10 minutes; after the time was complete, the mouse was removed and returned to its home cage.

Elevated Plus Maze. An elevated plus maze testing apparatus from Clever Systems Inc. was used for testing (CSI-MZ-EP-M). It has two open arms with a combination measurement of (CSI-MZ-M: Open Arm 7 cm width, 69 cm length, and 0 cm height) and two closed arms with a combination measurement of (CSI-MZ-M: Open Arm 7 cm width, 30 cm length, and 15 cm height). Tracking was performed using Noldus Ethovision (Ethos XT 17). The mouse was placed into the open arm and allowed to explore for 10 minutes. The mouse was then removed and returned back to its home cage. The elevated plus maze apparatus was cleaned with 75% alcohol between each individual test.

Hot Plate. The hotplate test was conducted using the Hotplate Analgesia meter from Columbus Instruments (serial #210028). Once the system was started and reached 55ºC, testing began. A timer was started when each mouse was placed inside the apparatus one by one and monitored for signs of thermal discomfort such as flicking of the tail and limbs, or combination of signs. Some mice did displace other signs such as immediate launching to the sides of the walls around the meter. Once signs were documented timing stopped and the mouse was removed to prevent any thermal injury. Any mouse was removed by 15 seconds if no signs were shown, to prevent thermal injury.

Tail Flick. Maze Engineers tail flick device was used to test each individual mouse. Mice were restrained in an A refrainer device (Maze Engineers). During the test the tail is free from the refrainer and placed over a light source. Once the light source is started, it heats to 25ºC, the mouse will move the tail, typically in a “flick” fashion. The tail flick device recorded the timing of how long the mouse kept the tail over the light source. All mice were removed after 15 seconds if no flick was present, to prevent any possible thermal injury.

Novel Spatial Object. Noldus Information Technology Inc. behavioral system Ethos XT 17 was used for tracking. A square box approximately 50 cm x 50 cm x 50 cm was used for the arena and one plastic square (5 cm x 5 cm x 5 cm) and one tin can (5 cm x 5 cm x 10 cm) was used as the objects during testing. The Novel object testing occurred over 6 days for 10 minutes per day. Day 0 was used for habituation to the for the arena with no objects. During days 1-4 objects were placed in the arena and the mouse was allowed to explore for 10 minutes. Testing was performed on Day 5 when one of the objects was moved to a new location. The object moved was counterbalanced across trials.

Contextual fear conditioning. Freeze Frame (Version 4.201) from Actimetrics software was used to score freezing behavior. The software worked in conjunction with Coulbourn habitest isolation cubicle (model # H10-24TA) and precision animal shocker (Serial #802313, Catalog # H13-15) to administer the foot shock protocol. The mice went through novel spatial object testing, prior to contextual fear conditioning, which provided several days of handling and transportation. Each mouse was transported one by one to the testing room on day 0 which was the contextual fear conditioning training day. During this, two-foot shock presentations (1 sec, 0.4 mA, 60 second inter-trial interval) were given after a 3 min baseline period. Animals were removed after being in the chamber for 5 min. The chamber was cleaned prior to each test experiment and wiped with 75% ethanol. The next day each mouse was placed back into the chamber without any shock presentations and monitored for freezing behavior across a 5 min period.

Statistical Analysis. All behavioral data is analyzed with a two-way factor ANOVA (genotype x sex), with an alpha set to 0.05. Tukey's HSD was used for post-hoc analysis.

RNA extraction, RNA-seq library construction, and data analysis. RNA was extracted from the hippocampus using the RNAeasy Mini Kit (Qiagen, Cat# 74104). Homogenized samples were subjected to the protocol provided by the manufacturer. The RNA concentrated on the silica membrane was eluted with RNase-free molecular biology-grade water. 150 ug total RNA collected from each tissue sample was shipped to Novogene Corporation Inc. for RNA-seq library construction. (Illumina). The libraries were sequenced on Novaseq 6000 platform with

150 bp paired-end mode (Illumina). Trim Galore (version [0.6.5]) was used to filter short and low-quality reads and trim adapter sequences from raw reads. Clean reads were mapped to the mm10 genome and quantified expression using STAR [(version 2.7.3a)]. The raw counts were employed to pair wisely and identify differentially expressed genes by R package DESeq2. Gene with larger than 1.2-fold change and adjusted p-value less than 0.05 were considered significant. R package glmmSeq was used to perform mix-model analysis and address Egr1-KO-dependet significant genes (adjusted p-value < 0.05).

GO analysis.

Gene Ontology (GO) analysis was performed and visualized using R packageclusterProfiler (v4.4.4). Default parameters were used for the enrichment analysis for biological process (BP). The resulting GO terms and the corresponding p-values were then processed using R package rrvgo to reduce GO term redundancy.

2.4 Results

Behavior changes observed in Nestin-Cre and Egr1cKO mice

Although previous research has extensively studied the behavioral changes of mice with *Egr1* complete knockout, no data has been collected on the effects associated with *Egr1* loss in neuronal lineage cells. In this study, we included (n=10 per sex/group) mice with four different genotypes ($Egr1^{f}Nes^{cre+}$, $Egr1^{f}Nes^{cre-}$, $Egr1^{wt}Nes^{cre+}$, and $Egr1^{wt}Nes^{cre-}$), to investigate the similarities and differences in the impact of the Nestin-Cre driver, Cre, and the loss of *Egr1* gene. As shown in **Fig. 1**, we conducted a behavioral test battery to evaluate pain sensitivity, anxiety-like behavior, and learning and memory.

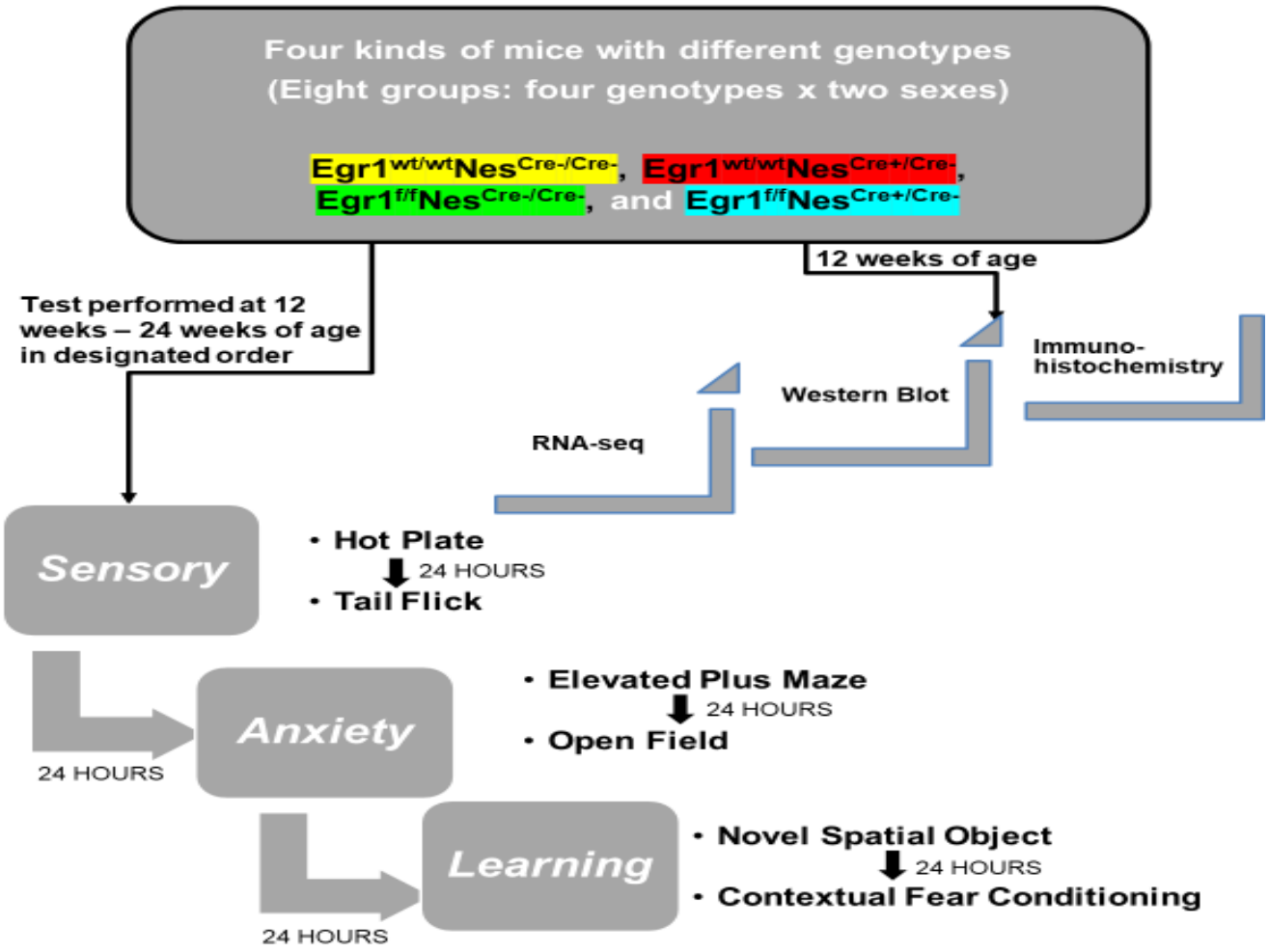


Figure 2.1 Diagram of experimental design and behavioral tests. Experimental design for gene expression and behavioral test scheme to explore the role and mechanism of Egr1 in Nestin-Cre driving conditional knockout mouse model.

The tail flick test measures the sensitivity of A-delta fibers with a mild and short-lasting nociceptive stimulus, while the hot plate test measures the sensitivity of C fibers with a more intense and enduring nociceptive stimulus[18]. For the hot plate test and tail flick (**Fig. S2A&B**), no difference was observed among the eight groups, suggesting all pathways are still physiologically intact and there were no changes in pain responses. The open field test measured exploratory anxiety-like and locomotor behavior. None of the eight groups showed any difference in time spent in the center of the open field (**Fig. S2C**); however, the total distance traveled during the open field test did have a significant interaction between genotype and sex ($F(3,70) = 5.15$, $p < 0.05$). Post hoc testing with Tukey's revealed the female group $Egr1^{f}Nes^{Cre-}$ (6525.99 $\pm$ 875.2 cm) had a ~4- 5 fold higher distance traveled in open field than female groups

$Egr1^{wt}Nes^{Cre-}$ (1524.996 ± 292.97 cm) and $Egr1^{wt}Nes^{Cre+}$ (1331.541± 99.69cm.) Female $Egr1^{f}Nes^{Cre-}$ mice were also significantly different from ($p<0.05$) male $Egr1^{f}Nes^{Cre-}$ mice (1915.66 ± 165.9 cm) and $Egr1^{wt}Nes^{Cre-}$ mice (2034.922 ± 328.72 cm) with an ~3-fold difference in distance traveled (**Fig. 2A**). As an additional measure of exploratory anxiety, we used the elevated-plus maze. This test measures a conflict between exploratory risks in the open arm vs. the safety signals of the closed arms. There was a significant interaction between genotype and sex ($F(3,72) = 3.81$, $p < 0.05$). Post hoc analysis with Tukey's found that female $Egr1^{wt}Nes^{Cre-}$ mice (294.82± 75.58 seconds) spent 2.8-fold more time in the open arms compared to the male knockout group- $Egr1^{f}Nes^{Cre+}$ (103.39 ± 20.98 seconds). We did not identify any within sex effects of knockout (**Fig. 2B**). Together, these data identify that neuron-selective deletion of *Egr1* within sex did not alter anxiety associated behavior, it did alter the relationship between activity levels and anxiety between the sexes.

To determine perceptual learning and recognition memory, we performed the novel object spatial recognition tests, which revealed no significant effect regardless of sex or genotype and demonstrated that spatial awareness and object recognition are not altered (**Fig. S2D**). We conducted contextual fear conditioning to examine the process of learning to associate aversive stimuli with contextual memory. Genotype affected the amount of time animals froze across shock exposure in a sex specific manner with significant interaction $F(3, 32) = 4.194$, $p<.05$. Post hoc analysis did not indicate a specific relationship in the groups. The $Egr1^{f}Nes^{Cre+}$ male group was the only group with a decrease in freezing percentage after the second shock (**Fig. 2C**). All groups had increased freezing across training. Freezing in the context of the absence of shock was used to test associative learning. There was a significant interaction between sex and genotype on contextual freezing $F(3, 71) = 3.557$, $p<.05$, as well as a main effect of sex $F(1, 71) = 50.37$, $p<.001$, and genotype $F(3, 71) = 8.023$, $p<.0001$. (**Fig. 2D**). Post hoc analysis with Tukey's identified the following differences within sex. $Egr1^{f}Nes^{Cre+}$ males had a higher percentage of freezing in the context than the $Egr1^{f}Nes^{Cre-}$ males ($p < 0.01$) Within females, $Egr1^{f}Nes^{Cre+}$ also froze more in the context than $Egr1^{wt}Nes^{Cre-}$ ($p < 0.01$). Overall, females froze less than males when returned to the context in which they were trained ($p < 0.05$).

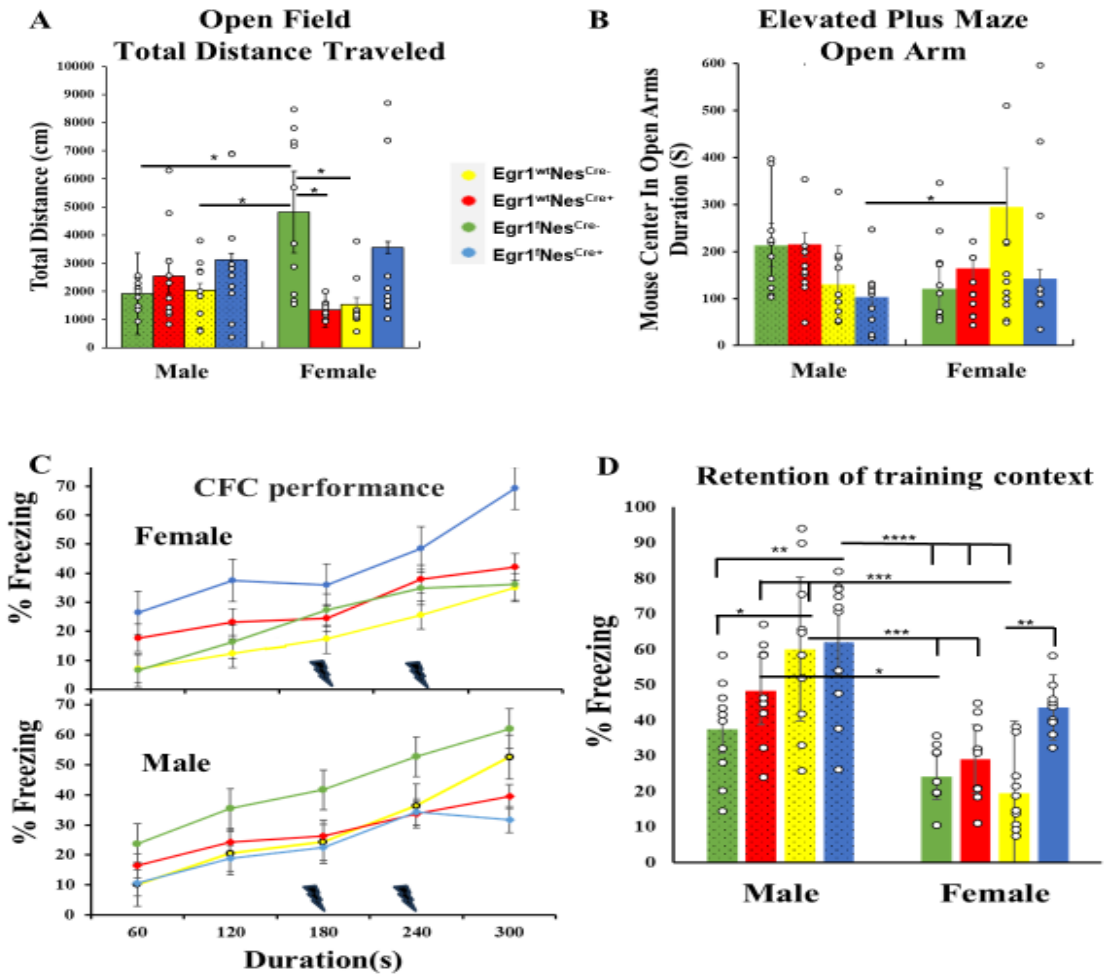


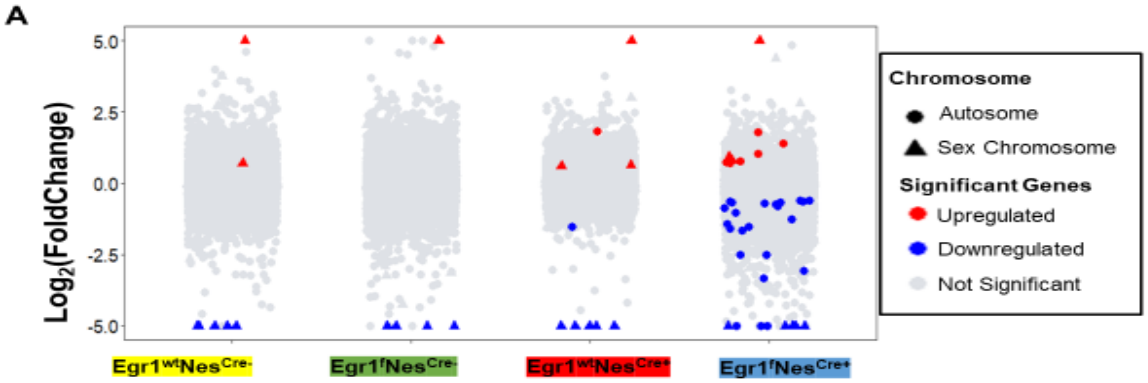
Figure 2.2 Behavioral test results for male and female mice of four genotypes. (**A**) Open Field Total distance traveled (cm) bar chart from males and females, with significance bars and * shown for sex and genotype. (**B**) Elevated Plus Maze Open Arms bar chart of time spent in open arms (s) for males and females. Significance is marked with a bar and * between groups. (**C**) Contextual Fear Conditioning line chart on a training day for freezing (%) of females (upper panel) and males (lower panel) only. Each point represented the mean during that minute of training in the CFC test. Lighting denotes the times that a shock was administered during the test. (**D**) Contextual Fear Conditioning freezing response (%) first day after training for both males and females. Bars represent means for genotypes over the entire freezing session. Significance above groups between sex and genotype along with * to denote significance level. The data for each time point in each test was derived from 10 individuals per group and presented as means ± SEM. Each bar is separated by sex and then genotype within sex and represented by color as well as males have textured bars. * denotes P-value that is significant, with different amounts of stars equating to different levels of P values, *p < 0.05, **p<0.005, *** p<0.001, and ****p<0.0001.

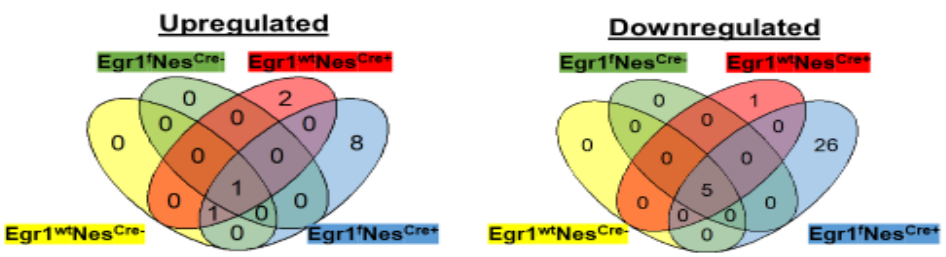
Sex differences in gene expression amplified in Egr1cKO mice

In addition to cataloging behavioral effects of neuron specific Egr1cKO and Nestin-Cre,

we examined their hippocampal gene expression patterns given the previously published reports of whole-body KO resulting in learning impairment. To accomplish this, RNA-seq was conducted in triplicates using adult hippocampal tissues obtained from the eight groups of mice. Upon analyzing the transcriptomic data using Principal Component Analysis (PCA), we found that the twenty-four samples could be separated into two groups based on their gene expression profiles. The female mice were positioned on the left upper side of the graph, while the males were positioned on the right (**Fig. S1A**). Pearson's correlation of gene expression across samples demonstrated sample variation within groups, but neither sex nor genotype is a good clustering parameter (**Fig. S1B**). We next performed pairwise comparisons for four genotypes separately to identify genes associated with sex-specific hippocampal expression. For wild-type mice ($Egr1^{wt}Nes^{Cre-}$), a total of 7 genes with 5 downregulated and 2 up-regulated in females (**Fig. 3A**). The numbers of differentially expressed genes (DEGs) involving sex-related difference were 6 in $Egr1^{f}Nes^{Cre-}$, 10 in $Egr1^{wt}Nes^{Cre+}$, but increased to 41 in $Egr1^{f}Nes^{Cre+}$ mice. The majority of DEGs identified between the two sexes were Egr1cKO-specific, and ten out of a total of 44 DEGs identified were derived from sex chromosomes (**Fig. 3B**). Only 1 gene is up-regulated, and 5 genes down-regulated in females were found to be shared by all four genotypes. These shared genes are all from sex chromosomes, such as Kdm5d on the Y chromosome and Xist on the X chromosome (**Fig. 3C**).

To further examine whether the small sample size (n=3) resulted in such a few DEGs detected, we divided twenty-four mice into two groups based on sex to perform pair-wise comparison. The increasing group size (n=12) did not yield much more DEGs, with only 18 DEGs determined (Fig. S3A). Although the majority of them show consistent expression profiles within sex groups (Fig. S3B), significant variation within female group was observed for two genes encoding for transporters, the sodium bicarbonate cotransporter gene (SLC4A5) and transthyretin (TTR) gene which transports vitamin A and thyroxine. Therefore, these two genes should be excluded from the sex-linked DEG list. Altogether, we concluded that only a limited number of genes are differentially expressed between male and female mouse hippocampi. Such a small sex difference is primarily driven by genes on sex chromosomes. However, the loss of *Egr1* gene leads to an amplification of sex-related differences in the hippocampus for approximately two dozen genes on autosomes.





C

Gene Name	Symbol	Chromosome	Egr1wtNesCre-	Egr1fNesCre-	Egr1wtNesCre+	Egr1fNesCre+
TSC22 domain family member 3	Tsc22d3	X	T			
eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	Eif2s3x	X	T	T		T
lysine (K)-specific demethylase 6A	Kdm6a	X		T		
inactive X specific transcripts	Xist	X	T	T	T	T
lysine (K)-specific demethylase 5D	Kdm5d	Y	T	T	T	T
eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	Eif2s3y	Y	T	T	T	T
	Gm29650	Y	T	T	T	T
ubiquitously transcribed tetratricopeptide repeat containing, Y-linked	Uty	Y	T	T	T	T
DEAD box helicase 3, Y-linked	Ddx3y	Y	T	T	T	T

Figure 2.3. Gene expression changes associated with sex difference. (**A**) Volcano plots for pairwise analyses between two sexes for four genotypes. DEGs were defined as genes with FC ≥1.2 and adjusted P value ≤0.05. Blue dots indicate DEGs down-regulated in males. Red dots indicate DEGs up-regulated in males; Grey dots indicate no DEGs. (**B**)Venn diagram showing the overlapped DEGs identified in pairwise comparisons. Corresponding genotypes were shaded with represented color (blue for $Egr1^{f}Nes^{cre+}$, green for $Egr1^{f}Nes^{cre-}$, red for $Egr1^{wt}Nes^{cre+}$and yellow for $Egr1^{wt}Nes^{cre-}$). (**C**) A list of DEGs on sex chromosomes.

Aberrant gene expression associated with Nestin-Cre, Flox-p, and Egr1cKO

To understand the effect of the Nestin-Cre driver and Flox-p on hippocampal expression, we first kept male and female RNA-seq data separately to perform pairwise comparisons (Fig. S4). Interestingly, no gene was found to be differentially expressed in male groups, and only 6 DEGs were identified in female groups with significant Fold Change (FC) and adjusted p-values below 0.05. Compared with $Egr1^{wt}Nes^{Cre-}$, four genes were upregulated in female $Egr1^{f}Nes^{Cre-}$,

including MFSD2 Lysolipid Transporter A (Mfsd2a, FC = 1.67, p= 0.044), Perilipin 4 (Plin4, FC = 3.83, p= 0.042), Solute Carrier Family 2 Member 1(Slc2a1, FC = 1.63, p= 0.045), and Xanthine Dehydrogenase (Xdh, FC = 2.42 , p= 0.042). Compared with $Egr1^{wt}Nes^{Cre-}$, Heat shock protein beta-8 (Hspb8, FC = 1.57, p= 0.045) was upregulated, and TCDD Inducible Poly (ADP-Ribose) Polymerase (Tiparp, FC = 1.83, p= 0.003) was downregulated in female $Egr1^{wt}Nes^{Cre+}$. Worthy of mentioning, Hspb8 and its α-crystallin domain might act as a pleiotropic pro-survival factor in the adult hippocampus[19]. TIPARP is an enzyme involved in molecular cascades regulating structural changes in synaptic connections and correcting distribution and number of GABAergic neurons[20]. Very few DGEs identified with FCs less than two folds and/or p-values on the edge of thresholds suggest that the presence of Floxp or Nestin-Cre driver may have an impact but subtle on female hippocampal functions.

We next focused on the transcriptomic analysis of Egr1cKO mice. We observed that the expression of *Egr1* gene is highly expressed in wild type mice but abolished in the hippocampi of both female and male mice (**Fig. 4A**). To validate the changes of *Egr1* expression at protein level, immunohistochemistry using anti-EGR1 antibody was performed on brain sections obtained from $Egr1^{f}Nes^{cre+}$ and $Egr1^{wt}\,Nes^{cre-}$ adult mice (**Fig. 4B**). EGR1 proteins are highly expressed in the hippocampus in $Egr1^{wt}\,Nes^{cre-}$ mice but substantially diminished in that of $Egr1^{f}Nes^{cre+}$ mice. Further western blot also demonstrated that the level of EGR1 proteins was significantly reduced in the hippocampus of Egr1cKO mice (**Fig. 4C**). Considering the similar transcriptomic profiles observed in both sexes of wild type mice, we divided the 24 mice into two groups: 18 with high *Egr1* expression and 6 with the loss of *Egr1* in cell lineages expressing Nestin gene. Pair-wise comparison determined 12 genes upregulated and 19 genes downregulated in Egr1cKO hippocampus (**Fig. 4D**). Heatmap of the expression profiles of these genes indicated that females show more striking and consistent changes than male counterparts (**Fig. 4E**).

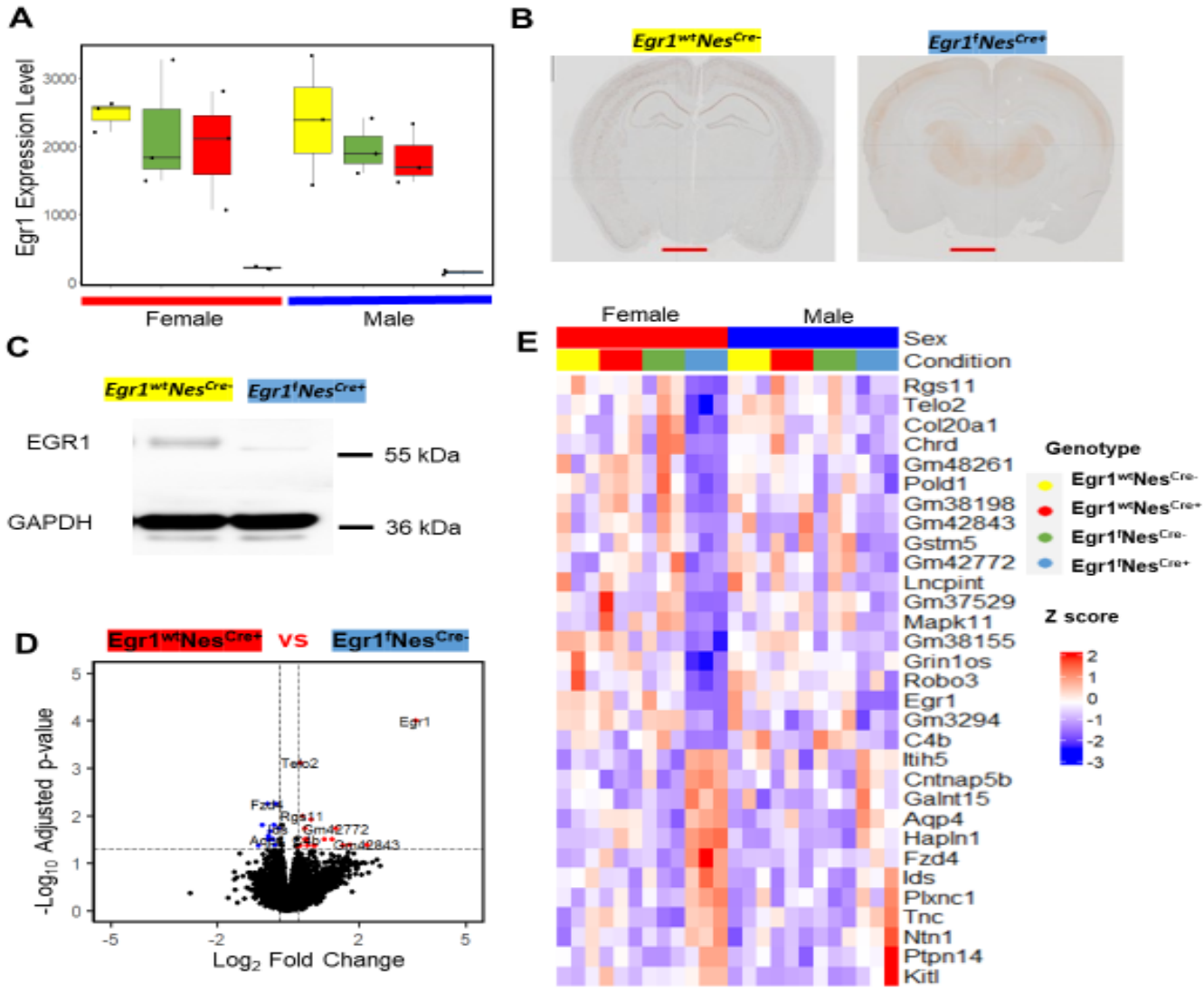


Figure 2.4. The expression of *Egr1* mRNA and EGR1 protein in the hippocampus of Egr1cKO mice and differential expressed genes associated with Egr1cKO identified by pairwise comparison. (**A**) Box plots for *Egr1* expression levels in mice of four kinds of genotypes determined by RNAseq. (**B**) Immunohistochemistry staining of EGR1 protein expression in mouse brain (coronal view, scale bar = 2mm). (**C**) Western blot of EGR1 protein expression in mouse hippocampus, GAPDH serves as internal control. (**D**) Volcano plot for pairwise analysis between mouse hippocampi with and without Egr1cKO. DEGs were defined as genes with FC ≥1.2 and adjusted P value ≤0.05. Blue dots indicate DEGs down-regulated in *Egr1* wildtype controls. Red dots indicate DEGs up-regulated in *Egr1* wildtype controls; Black dots indicate no DEGs. (**E**) Heatmap of gene expression profiles for DEGs identified.

In this study, four factors (sex, flop, Nestin-Cre, and the loss of *Egr1* expression) may contribute to hippocampal gene expression changes. Since pairwise comparison is not sufficient

to scrutinize the influence of these four factors simultaneously, we adopted a mixed effect model to analyze the hippocampal gene expression profiles from the mice of all four genotypes (**Fig. 5A**). In this mixed model, the combination of Flox-p and Nestin-Cre would lead to the loss of *Egr1*. Interestingly, nine genes, including *Egr1*, were shown to be under the influence of Flox-p, Nestin-Cre, and Egr1cKO (**Fig. 5B**). Five genes were determined to be specifically associated with Flox-p genotype, and 18 genes were linked to Nestin-Cre (**Fig. 5B&C**). Further PCA analyses using the expression patterns of these genotype-linked genes are sufficient to separate the mice into groups corresponding to their genotypes (**Fig. 5 D&E**). The PC1 variances, the amount of variability in a data set that can be attributed to first principal component, are 79% for floxP-linked genes and 56% for 18 Nestin-Cre linked genes. Therefore, pairwise comparisons are straightforward in identifying expression differences between two groups. Still, a mixed effect model is powerful to reveal underlying changes associated with multiple factors in a complicated experimental design.

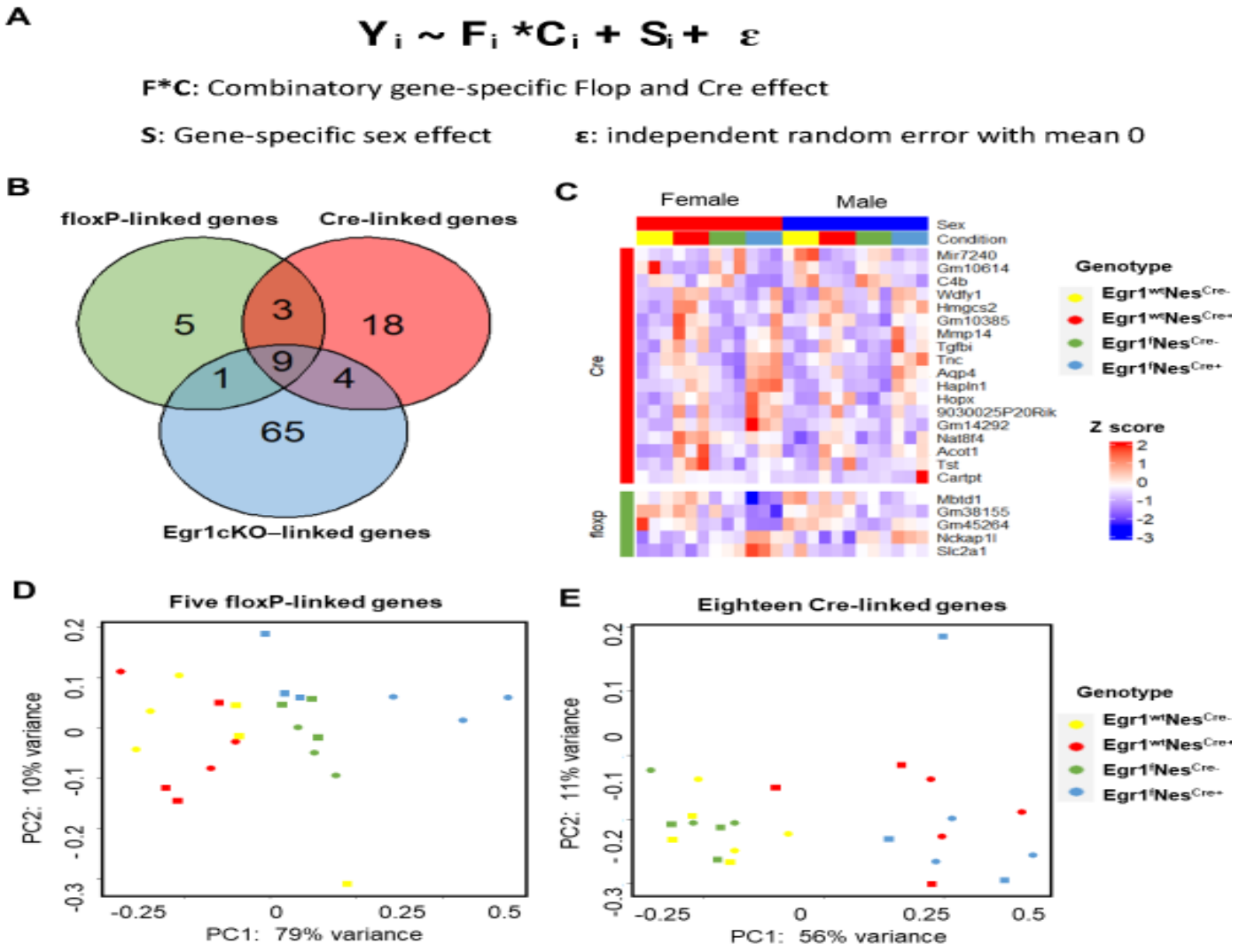


Figure 2.5. **Differential gene expression analysis using a mixed-effects model.** (**A**) Formula of mixed-effects model. (**B**)Venn diagram showing the overlapped DEGs identified. (**C**) Heatmap of gene expression profiles for DEGs identified to be linked with Flox-p or Nestin-Cre. (**D&E**) PCA analyses for five Flox-p-linked (**D**) and 18 Nestin-Cre-linked genes(**E**).

Lastly, we examined the influence of *Egr1* loss using mixed model. A total of 79 genes were identified, with only six overlapping with 31 DEGs determined in pairwise comparison. The overlapped six genes in the two lists include *Egr1*, Mitogen-activated protein kinases 11 (*Mapk11*), Contactin-associated protein like 5-2 (*Cntnap5b*), Iduronate 2-sulfatase (*Ids*), Tyrosine-protein phosphatase non-receptor type 14 (*Ptpn14*), and Gm42772. As one of the four

p38 MAPKs, MAPK11 (p38β) plays an important role in response to extracellular stimuli. *The cntnap5b gene encodes an extracellular matrix protein that functions* as a cell adhesion molecule and has been associated with dyslexia, a learning disability[21]. PCA analyses were performed using the expression profiles of two gene lists, the 31 DEGs derived from pairwise comparison and the 79 genes derived from mixed model (**Fig. 6A&B**). We found both gene sets were sufficient to separate the mice into two groups with or without *Egr1* loss. This result indicated that pairwise comparison and mixed model are useful for identifying genes that might be associated with *Egr1* loss.

Further, GO analysis for biological processes indicated that the union set of 104 DEGs linked to *Egr1* conditional knockout were enriched for Wnt signaling pathway, regulation of protein serine/threonine kinase activity, extracellular matrix components, axon guidance, and development of nervous system (**Fig. 6C**). Interestingly, these biological processes are not independent of each other. Wnt signaling is a critical pathway regulating neuronal extracellular matrix expression and axon terminal expansion[22, 23]. On the other hand, the composition of extracellular matrix could dictate Wnt expression[24]. Extracellular matrix molecules are key components in the formation of axonal tracts, and both biological processes are involved in neuronal maturation and synaptogenesis[25]. EGR1 can activate the Wnt/beta-catenin, promoting oncogenesis in tumoral cells[26] but induces apoptosis in leukemia cells[27]. Together with these findings, our results support that *Egr1* gene may regulate Wnt signaling in different types of cells to achieve distinct functions. Altogether, our study reported the indispensable role of *Egr1* in regulating Wnt signaling, extracellular matrix, and axon guidance in neuronal cell lineage. This provides an important mechanistic view of how *Egr1* contributes to synaptogenesis, neuron migration, and maturation.

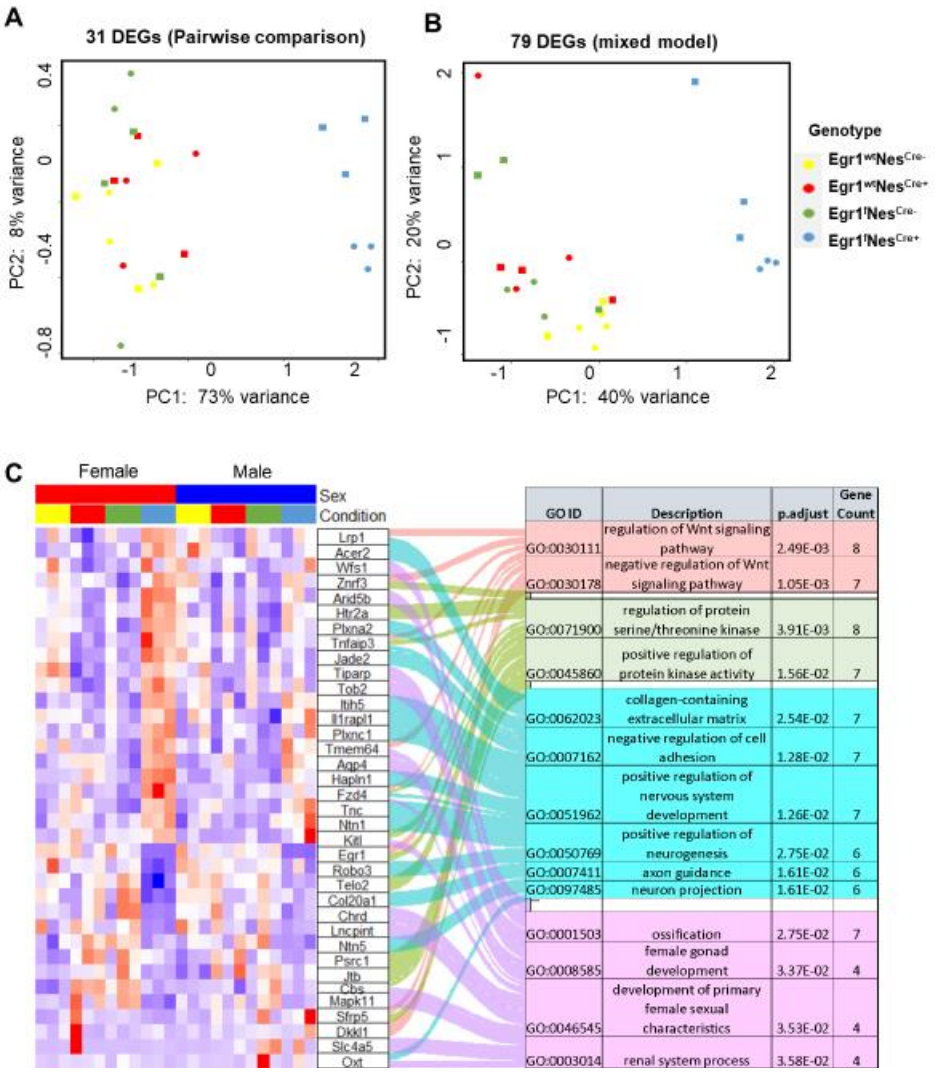


Figure 2.6. Differential expressed genes associated with Egr1cKO. (**A&B**) PCA analyses for 31 DEGs identified by pairwise comparison (**A**) and 79 DEGs identified by the mixed model (**B**). (**C**) Heatmap of gene expression profiles for DEGs identified to be enriched in GO biological processed.

2.5 Discussion

The Nestin-Cre transgenic mice have been widely used to study gene functions in the central nervous system. In this study, Nestin-Cre driver is able to successfully knockout *Egr1* expression in hippocampal cells. Both *Egr1* loss and Nestin-Cre driver have been reported to be associated with behavior abnormalities in transgenic mice. In this study, for mice of four genotypes related to Egr1cKO driven by Nestin-Cre, we performed a battery of behavior tests to monitor their responses to stress, learning, and memory.

Complete Egr1KO mice have abnormal spatial memory and decreased ability to associate shock with context in fear conditioning. Nestin-Cre driver only mice have been characterized to have reduced growth rates and altered behavioral responses to stress[28]. In this study using Egr1cKO driven by Nestin-Cre, we observed that pain responses and spatial novel object testing were not altered. Egr1cKO had some exploratory anxiety associated with differences between sex, but these are difficult to interpret given the effects on activity in the open field. There was a clear behavioral difference in training and testing contextual fear conditioning. The males froze more in the context than females, and the Egr1cKO group in both sexes froze more than controls. The Wnt signaling pathway in the hippocampus has been identified as a key driver of social avoidance in male mice following defeat stress[29], along with other neuropathic disorders, specifically in the hippocampus[30]. Our study suggests that Egr1cKO could lead to abnormal Wnt signaling, causing behavioral changes that influence anxiety. Future studies should be conducted to define specific pathways linked with Egr1 and Wnt signaling pathways to further understand the links between molecular and behavior changes.

Our IHC and western blot results demonstrated that EGR1 was largely depleted from the Egr1cKO brain using the Nestin-Cre Lox-p system. However, such a knockout is not hippocampal specific, and brain regions other than the hippocampus can also participate in behavior control. This limits our ability to interpret behavior changes observed with hippocampal gene expression analysis alone. In addition, *Egr1* is known to have a decreased expression in the brains of schizophrenia patients[5, 31]. However, the blood samples from the sample patients with lower brain *Egr1* expression showed an increased expression of *Egr1*[32, 33]. Such a phenomenon could result from the attempt of our bodies to compensate for lower brain *Egr1* expression. Thus, other unknown compensatory mechanisms for the decreased expression of *Egr1* may exist and warrant further investigation.

To our knowledge, this is the first study systematically analyzing transcriptomes of Egr1cKO mice together with Cre, Flox-p, and wild type controls. Between the two sexes, only a handful of genes were found to be differentially expressed in mouse hippocampus, and the presence of Nestin-Cre or Flox-p loci did not introduce substantial changes. Notably, for 22 out of 24 mice used in this study, we have performed transcriptomic analysis for pituitary tissues as well. A total of 217 genes were identified to be differentially expressed between male and female pituitary tissues in wild type controls, and such a number increased to approximately

2,000 in $Egr1^{f}Nes^{Cre+}$ mice. Nestin-Cre and Flox-p alone also showed significant influence on pituitary sex-linked gene expression patterns. Such a tissue-specific difference in sex-linked gene expression patterns observed in our studies raised an issue that some conclusions derived from transgenic mice may not be able to generalize to both sexes and across tissues.

Despite using both a pairwise comparison approach and a mixed effect model, we were only able to identify a small number of DEGs in hippocampus associated with distinct genotypes. In addition, the small fraction of significant genes detected by both approaches indicated that the transcriptomic influence of *Egr1* loss in neuronal lineage cells is subtle at whole tissue level or covered by the transcripts derived from other types of cells present in hippocampus. Single cell RNAseq in future may help to provide a more complete picture. Regardless, for Egr1cKO, GO term analysis identified several major biological processes enriched for the list of differentially expressed genes. Future studies would be desired to better understand the functions of *Egr1* in distinct neuronal types when animals perform diverse memory tasks.

2.6 Conclusions

Altogether, our results support that Nestin-Cre driver can efficiently remove *Egr1* genes in neuronal lineage cells in hippocampal cells. RNA-seq analysis revealed that Egr1cKO disrupted Wnt signaling and axon guidance pathways. In addition, sex differences in behavior and gene expression were observed in the Egr1cKO mice, and female mice are more sensitive to the loss of *Egr1* gene.

Declaration of interest

The authors declare no competing financial interests and that no conflict of interest could be perceived as prejudicing the impartiality of the research reported.

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Ethics approval and consent to participate

All procedures were performed following national and international guidelines and approved by the institutional board at Virginia Tech. The study is in accordance with National Institute of Health guidelines.

Availability of data and materials

RNA-seq datasets generated in this study have been deposited in NCBI Gene Expression Ominibus (GEO) under accession number GSE232396.

Author contribution statement

C S and H X conceived the experimental design. C S and M L were responsible for animal breeding and genotyping PCR. M L was responsible for the RNA isolation. C S was responsible for all behavioral experiments with guidance from G H and T J. C S and X X were responsible for immunohistochemistry. Y Z and Y L were responsible for mRNA-Seq data processing and conducted the statistical evaluation. C S, G H, T J, and H X interpreted the results and drafted the manuscript. All authors discussed the results, read, and edited the manuscript, and approved the final manuscript.

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Chapter 3 Sex-Linked Growth Disorder and Aberrant Pituitary Gene Expression in Nestin-Cre-Mediated *Egr1* Conditional Knockout Mice

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3.1 Simple Summary

Reduced growth hormone levels were observed in mice without the *Egr1* gene and mice carrying a Nestin-Cre driver. It remains unknown why these two strains of mice share a similar phenotype and whether the dysregulation of hormone production in the pituitary occurs via the same mechanism. Previous studies have shown conflicting results regarding the effectiveness of Nestin-Cre in driving gene knockout in the pituitary. In this study, we found that while the Nestin-Cre driver successfully removed *Egr1* expression in neuronal lineage cells, it did not effectively remove it from all sections of the pituitary. Moreover, the Nestin-Cre driver alone caused growth abnormalities in the mice and influenced the expression of genes related to growth factors in the pituitary. Sex differences were also observed in the mice that exhibited these growth and gene expression abnormalities, with female mice being more sensitive to the presence of Nestin-Cre and the loss of *Egr1*. Overall, this study highlights the limitations of the Nestin-Cre driver in removing *Egr1* from pituitary cells and provides insights into the impact of Nestin-Cre on growth and gene expression, particularly in relation to concerning sex differences.

3.2 Abstract

Genes that regulate hormone release are essential for maintaining metabolism and energy balance. *Egr1* encodes a transcription factor that regulates hormone production and release, and a decrease in growth hormones has been reported in *Egr1* knockout mice. A reduction in growth hormones has also been observed in Nestin-Cre mice, a model frequently used to study the nervous system. Currently, it is unknown how *Egr1* loss or the Nestin-Cre driver disrupts pituitary gene expression. Here, we compared the growth curves and pituitary gene expression profiles of Nestin-Cre-mediated *Egr1* conditional knockout (*Egr1*cKO) mice with those of their controls. Reduced body weight was observed in both the Nestin-Cre and *Egr1*cKO mice, and the loss of *Egr1* had a slightly more severe impact on female mice than on male mice. RNA-seq data analyses revealed that the sex-related differences were amplified in the Nestin-Cre-mediated *Egr1* conditional knockout mice. Additionally, in the male mice, the influence of *Egr1*cKO on pituitary gene expression may be overridden by the Nestin-Cre driver. Differentially expressed genes associated with the Nestin-Cre driver were significantly enriched for genes related to growth factor activity

and binding. Altogether, our results demonstrate that Nestin-Cre and the loss of *Egr1* in the neuronal cell lineage have distinct impacts on pituitary gene expression in a sex-specific manner.

Keywords: sex difference; *Egr1*; Nestin; RNA-seq; pituitary; growth

3.3 Introduction

The pituitary gland is an organ that controls endocrine homeostasis by releasing a number of several hormones to regulate growth, metabolism, stress responses, and the reproductive system. It can be divided into three sections: the anterior, intermediate, and posterior lobes [1]. Each section comprises distinct types of pituitary-specific cells characterized classically by the hormones they synthesize. Recent single-cell transcriptome profiling of the rat anterior pituitary identified folliculostellate cells, five hormone-producing cells, endothelial cells, and blood cells [2]. In the mouse pituitary, a single-cell analysis led to the identification of a "multi-hormonal" cell cluster that expressed growth hormone (GH), prolactin (PRL), and thyroid-stimulating hormone (TSH) simultaneously [3]. The well-characterized gonadotropes in the pituitary gland produce gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). These hormones control gonadal function and development in both sexes. LH and FSH are heterodimeric glycoproteins that share a common α-glycoprotein hormone subunit but have distinct β subunits. The expression of the LH-$_\beta$ subunit (LH_β) is regulated by three key transcription factors: steroidogenic factor 1 (sf1), pituitary homeobox factor 1 (Pitx1), and early growth response factor 1 (*Egr1*) [4–8]. The *Egr1* gene encodes a transcription factor with three zinc finger binding domains that recognize the consensus GC-rich genomic sequence 50 -GCG(T/G)GGGCG-30, regardless of the methylation status of the cytosine [9]. As an immediate early gene, *Egr1* can be rapidly and transiently induced in response to various environmental stimuli [10–12]. Activation of the *Egr1* gene does not require de novo protein synthesis. During cell specification, the EGR1 protein has been found to serve as a critical epigenetic regulator that recruits a DNA demethylation enzyme to remove cytosine methylation for downstream gene activation [13]. In the anterior pituitary, *Egr1* induction via gonadotropin-releasing hormone (GnRH) plays a critical role in regulating the synthesis of LHβ in gonadotrope cells [4,14]. A recent single-cell analysis demonstrated that gonadotrope cells exposed to GnRH exhibit the concentration-dependent bimodal response pattern of *Egr1* expression [15]. Furthermore, as GnRH concentration increases, the proportion of *Egr1*-expressing cells increases accordingly. The functional role of *Egr1* in the

pituitary was demonstrated in transgenic mice with complete knockout (*Egr1*KO) of the *Egr1* gene [16]. The loss of the *Egr1* gene led to a deficiency in LH_{β} production and female infertility. These *Egr1*KO mice were of smaller stature than the control mice, and the size of the pituitary gland pituitary size was reduced in both sexes. It was determined that the *Egr1* gene affects the cell differentiation of the somatotrophs and consequently results in a lower level of GH [17]. Many pituitary hormones have sex-specific functions and are synthesized in response to physiologic stimuli related to sex differences. A recent transcriptomic study revealed that significant sex differences in pituitary gene expression can be detected before puberty in mice; they can be detected as early as postnatal day 12 and persist post-puberty at least until postnatal day 37 [18]. Pituitary cellular composition can differ according to sex as well [3]. The percentage of gonadotropes in the male pituitary is approximately twice that in the female pituitary, and this difference is accompanied by a relative predominance of somatotropes over lactotrophs. In the female rat pituitary, the *Egr1* gene promoter and first intron provide cis-regulatory elements that drive the cell type-specific expression of *Egr1* in LH_{β} expressing cells [19,20]. In the male rat, *Egr1* is expressed in a subset of somatotrophs and lactotrophs, and it is co-expressed with LH_{β} in gonadotrophs. Still, its transcription was not observed in pituitary precursor cells expressing TSH, adrenocorticotropic hormone (ACTH), or Sox2 [21]. Despite the important role of *Egr1* in the development of gonadotropes, the male *Egr1*KO mice did not show reproduction defects as striking as those observed in the female mice. This sex difference was speculated to be associated with the dynamic regulation of *Egr1* expression in gonadotrophs during the estrous cycle [16,17]. The contribution of the *Egr1* gene to pituitary sexual dimorphism remains poorly understood. The Nestin-Cre transgenic mouse is one of the mouse models most frequently used to study gene functions in the central nervous system. The Nestin gene encodes a member of the intermediate filament proteins, which are primary components of the cytoskeleton. It was initially discovered in neural stem cells but has also been located in several different tissues throughout the body, including the pancreas, small bowel, muscles, and pituitary [22]. One of the first studies using Nestin-Cre mice targeted the glucocorticoid receptor and found the Nestin-Cre mice to be smaller than the control mice and to exhibit lower anxiety responses [23]. Nestin-Cre transgenic mice consistently report higher anxiety thresholds and smaller body sizes [24]. This was speculated to result from the hypothalamic expression of the human growth hormone gene inserted downstream of the Cre recombinase. An early study on Nestin-Cre transgene expression found that it was

present in the embryonic pituitary as well as in the stem cells of the adult pituitary [25]. However, it is still unknown whether it can achieve high efficiency in Nestin-Cre-mediated recombination in pituitary cells. In this study, we utilized the Nestin-Cre driver to generate *Egr1* conditional knockout (*Egr1*cKO) mice without *Egr1* gene expression in their neuronal lineage cells. We monitored the growth rates and examined the sex-related differential gene expressions in the pituitary glands of the *Egr1*cKO mice, comparing them with those of the Nestin-Cre controls. Our aim was to gain insights into the potential interplay between the *Egr1* gene and the Nestin-Cre driver in the disruption of pituitary gene expression. Additionally, we sought to investigate the effectiveness of Nestin-Cre-mediated recombination in removing the *Egr1* gene specifically from pituitary cells.

3.4 Material and Methods

Animals

All animal experiments were performed in accordance with the Virginia Tech (Blacksburg, VA, USA) Institutional Animal Care and Use Committee guidelines. The Egr1 conditional knockout mouse strain (Egr1_tm1a_A04, C57BL/6N-Egr1/Tcp; MGI:5766027) was purchased from the Centre for Phenogenomics, Canada. The Nestin-Cre (B6.Cg-Tg (Nes-Cre)1Kln/J; Jackson Lab, #003771) was a kind gift from Dr. Michael Fox's lab. To create the Egr1cKO mice, a breeding scheme was implemented to cross individuals with varying degrees of floxed Egr1 alleles with heterozygous Nestin-Cre mice until the desired Nestin-Cre-driven Egr1cKO was achieved. The mice were maintained and bred in a 12 h light/dark cycle under standard pathogen-free conditions. Free food (Teklad Global 18% Protein Rodent Diet) and water access were provided.

Measurement of Growth

The weights of the Egr1cKO mice and their corresponding wild-type littermates, as well as those of the heterozygous Nestin-Cre positive mice and the controls, were measured for 12 weeks. Each data point represents the average of at least ten mice, and standard derivations (SDs) are indicated.

Genotyping PCR

The mice were genotyped at three weeks of age during the time of weaning. A small distal portion of the tail was removed and incubated at 55 ◦C overnight in DirectPCR lysis reagent (Viagen,

Cedar Park, TX, USA, cat# 101T) solution along with Proteinase K (Invitrogen, Carlsbad, CA, USA, cat# AM 2546). The next day, the sample was incubated at 80 ◦C for one hour to deactivate the proteinase K before preparing for PCR sampling. Genotyping PCR reactions were performed according to the Jackson Laboratory's protocol for Nestin-Cre and the Centre for Phenogenomics' protocol for Egr1-loxp. A GoTaq G2 Green Master Kit (Promega, Madison, WI, USA, cat# M7822) and Egr1 primers (Egr1KOGT-For:50 -GGG AGG GTT TGT TTT GAT GA-30 and Egr1KO-GT-Rev: 50 -CCA GCA CCC TAG TGGCTA CA-30) or Nestin-Cre primers (Nes-CRE SET R: 50 -TGC ATG ATC TCC GGT ATT GA-30 and Nes-CRE SET- F 50 -CGT ACT GAC GGT GGG AGA AT-30) were used to create an Egr1 or Nestin-Cre PCR mixture. Agarose gel (General Purpose LE, El Cajon, CA, USA, Cat# 20-101) electrophoresis was used to identify the PCR products. A GeneRuler 100 bp DNA ladder (ThermoFisher, Waltham, MA, USA, Cat# SM0241) was used to determine the PCR product sizes.

Immunohistochemistry (IHC)

The Egr1cKO mice and the controls were rapidly and deeply anesthetized with isoflurane (Vet One, Boise, ID, USA, cat# 502017) and perfused transcardially with 10% formalin into the left ventricle. The right ventricle was opened to allow for exsanguination. After cardiac arrest was confirmed, the mice went through cervical dislocation. The brain was exposed, and the maximum amount of bone was removed to allow for further tissue fixation in 10% formalin overnight. The pituitary was left in the Sella turcica and dissected away from the sphenoid bone. It was then fixed for 48 h in 10% formalin before being removed. Both tissues, once fixed, were sent to the pathology laboratory of the Virginia–Maryland College of Veterinary Medicine for embedment. Coronal slices of the tissues in the wax block were taken and sent back to the pathology laboratory. Both the whole brain and the pituitary gland went through an automotive system involving a Ventana Discovery Ultra machine (DAB Detection kit Cat#: 760-159) and secondary antibody OMap anti-Rb HRP Cat#: 760-4311, purchased from Roche Diagnostic, Indianapolis, USA. Both the whole brain and the pituitary gland were stained with anti-Egr1 rabbit antibody (41542, purchased from Cell Signaling Technology, Danvers, MA, USA). Images were acquired using a MoticEasy Scan Pro 6 slide scanner.

RNAscope

Additional tissue samples were taken from the slices of embedded tissue used for IHC. An RNAscope procedure was carried out in accordance with the Advanced Cell Diagnostics protocol for RNAscope® Multiplex Fluorescent Reagent Kit v2 Assay (Document Number 323100-USM). Briefly, the FFPE sections were baked at 60 ◦C for 1 h in a dry oven, deparaffinized, and incubated with hydrogen peroxide at RT for 10 min. After target retrieval, the sections were incubated with probes and put through three sequential amplifications followed by signal development. Probes (Mm-Egr1-C3 REF 423371-C3, Mm-Lhb-C2 REF 478401-C2, and Mm-Gh REF 445361) were used for RNAscope detection. The slides were counterstained with DAPI and mounted with Prolong Gold anti-fade mountant (Invitrogen, cat# P36930). Images were taken using a Zeiss LSM 880 confocal microscope from the Virginia Tech Fralin Imaging Center.

RNA Extraction, RNA-Seq Library Construction, and Data Analysis

RNA was extracted from the pituitary using an RNAeasy Mini Kit (Qiagen, Hilden, Germany, Cat# 74104). The homogenized samples were subjected to the protocol provided by the manufacturer. The RNA concentrated on the silica membrane was eluted with RNase-free molecular biology-grade water. A total of 150 ng of RNA was collected from each tissue sample and shipped to Novogene Corporation Inc., Sacramento, CA, USA. for RNA-seq library construction. The libraries were sequenced on the Hiseq 4000 platform using the 150 bp paired-end mode (Illumina, San Diego, CA, USA). Trim Galore (version 0.6.5) was used to filter short or low-quality reads and trim adapter sequences from raw reads. Clean reads were mapped to the mm10 genome, and expression was quantified using STAR (version 2.7.3a). The raw counts were employed to pair wisely identify differentially expressed genes using R package DESeq2. Genes with a greater than 1.5-fold change and adjusted p-value of less than 0.05 were considered significant. R package glmmSeq was used to perform a mixed-model analysis and assess significant Egr1KO-dependent genes (adjusted p-value < 0.05)

GO Analysis

A gene ontology (GO) analysis was performed and visualized using R package cluster Profiler (v4.4.4). Default parameters were used for the biological process (BP) enrichment analysis. The

resulting GO terms and corresponding p-values were then processed using R package rrvgo to reduce GO term redundancy.

Statistical Analysis

The data in the growth curves are expressed as mean ± SD. A Student's t-test was used to determine the significant differences between the two groups with the following critical values: * for $p < 0.05$ and ** for $p < 0.01$.

3.5 Results

Generation and Characterization of Egr1cKO Mice

Both Nestin-Cre and *Egr1*KO mice show impaired pituitary functions. To explore the commonalities and sex differences resulting from the Nestin-Cre driver and *Egr1* gene, a total of eight groups of mice with four different genotypes were included in this study (Figure 1A). Their genotypes are denoted as *Egr1*^{f}Nes^{cre+}, *Egr1*^{f}Nes^{cre-} *Egr1*^{wt}Nes^{cre+}, and *Egr1*^{wt}Nes^{cre-} in the text following Figure 3.1. Only heterozygous Nestin-Cre mice were used in this breeding scheme to ensure that only one copy of the Nestin-Cre driver would be present in the genome of the Nes-Cre+ mice the *Egr1*^{f} mice had two copies of *Egr1*-loxp to ensure the removal of the *Egr1* gene from the two paired chromosomes. We performed genotyping PCR reactions to determine the presence or absence of the Nestin-Cre driver and *Egr1*-loxp, respectively (Figure 3.1B). The Nes-Cre+ mice yielded a PCR product at 350 bp, while no PCR product could be obtained from the wild-type controls. Using primers provided by the Centre for Phenogenomics in Canada, PCR products with 102 bp and 378 bp were obtained from the homozygous *Egr1*^{f} mice and the wild-type controls, respectively.

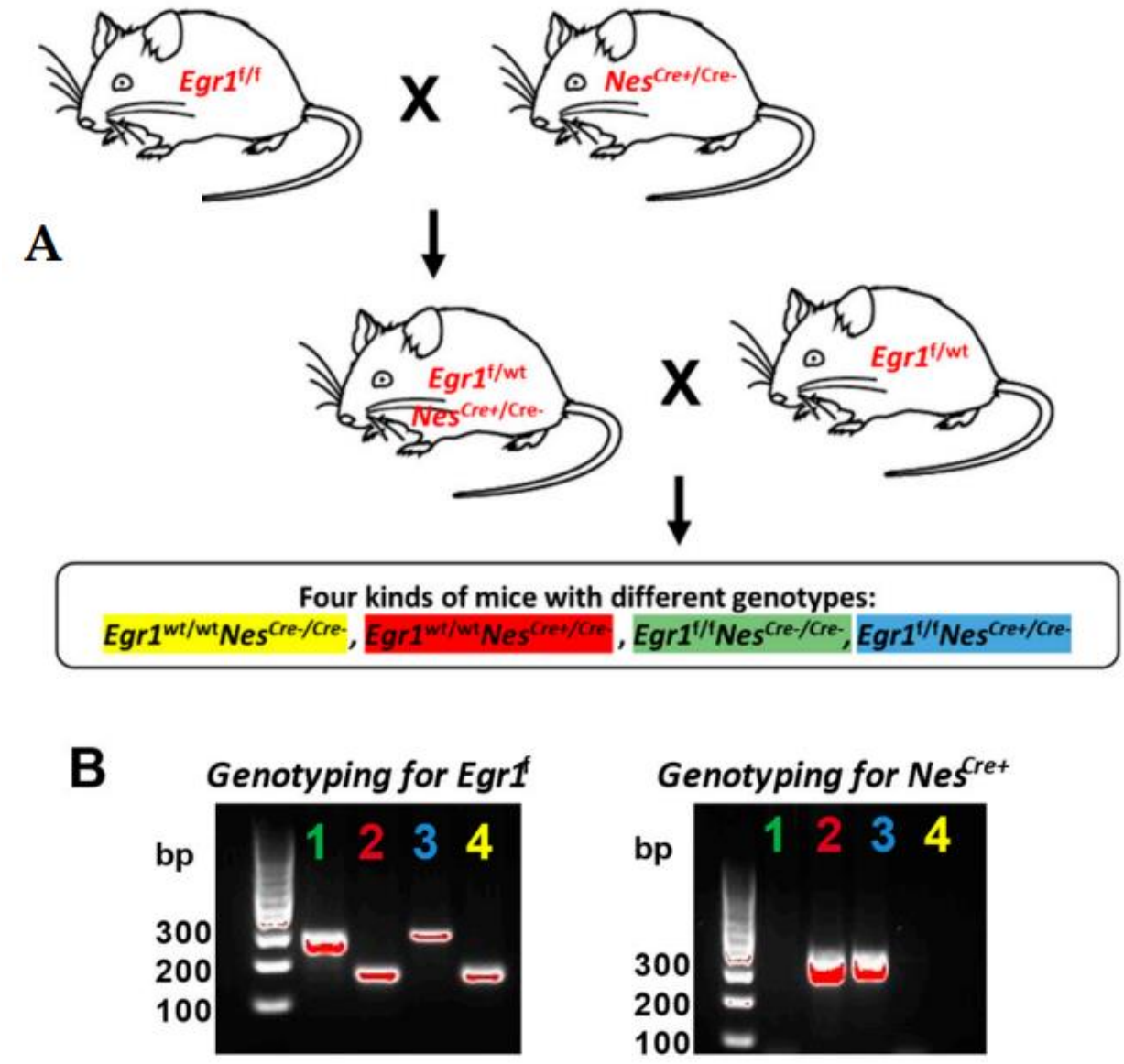

Figure 3.1 Generation of Egr1cKO mice. (A) Breeding scheme to obtain Egr1cKO mice. Four kinds of mice with different genotypes were included in this study, with $Egr1^{f/f}$ Nes^{Cre+} being the desired knockout genotype and the remaining genotypes being used as controls. The desired knockout genotype and the controls are all color coded throughout the paper. $Egr1^{f}$ represents a floxed Egr1 allele and Nes^{Cre+} represents the presence of Nestin-Cre in the loxp system, while Nes^{Cre-} represents its absence. **(B)** Genotyping PCR to validate the presence of Nestin-Cre or Egr1 in the loxp systems of the mice. Egr1f is 291 bp while wild-type is 165 bp; Nes^{Cre+} is 350 bp while Nes^{Cre-} has no band.

Growth Reduction Observed in Both Nestin-Cre and Egr1cKO Mice

Despite the known impact of the Nestin-Cre driver and the loss of the *Egr1* gene on body weight, no growth curve has been provided to demonstrate the progressive changes that occur in *Egr1*cKO and Nestin-Cre mice during postnatal development. Therefore, we monitored the eight groups of mice for twelve weeks after their birth (Figure 3.2). The difference the growth rate of the *Egr1*^{wt}Nes^{cre+} and that of the *Egr1*^{wt}Nes^{cre-} mice became significant with the approach of young adulthood and was consistent after eight weeks. Compared with the female mice, the males showed more significant differences, with a p-value ≤ 0.01 from 8 to 12 weeks. Compared with the controls of the same sex, reductions in body weight of around 12% and 8% were observed in the male and female *Egr1*^{wt}Nes^{cre+} mice, respectively. At the twelfth week, the average weight of a male *Egr1*^{wt}Nes^{cre+} mouse was 22.0 ± 1.98 g, while the average weight of a male *Egr1*^{wt}Nes^{cre-} mouse was 25.0 ± 1.87 g. The average weight of a female *Egr1*^{wt}Nes^{cre+} mouse was 19.0 ± 1.30 g, while the average weight of a female *Egr1*^{wt}Nes^{cre-} mouse was 20.7 ± 1.82 g.

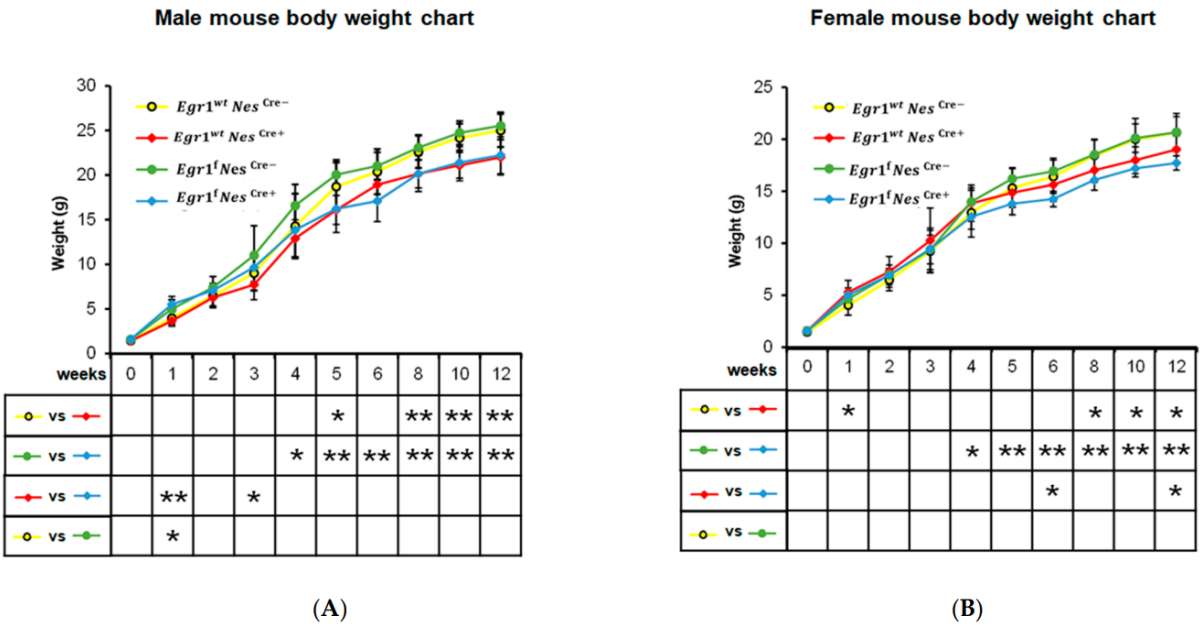

Figure 3.2 Growth curves for **(A)** male and **(B)** female mice of four genotypes from birth to 12 weeks. The data for each time point were derived from 10 individuals and are presented as means ± SEM. A group comparison was performed using a Student's t-test. * denotes p-value < 0.05 and ** denotes p-value < 0.01.

A previous study indicated that the body weights of both male and female homozygous *Egr1*KO mice were significantly and consistently lower than those of normal controls of the same sex at ages older than two weeks [17]. In our study, the conditional *Egr1* knockout delayed such differences between the sexes. Starting at four weeks of age, the *Egr1*^{f}Nes^{cre+} and *Egr1*^{f}Nes$^{cre–}$ mice exhibited reduced body weight steadily and with increasing significance. In the twelfth week, the average weight of a male *Egr1*^{f}Nes^{cre+} mouse was 22.2 ± 2.07 g, and the average weight of a male *Egr1*^{f}Nes$^{cre–}$ mouse was 25.5 ± 1.53 g, i.e., a greater than 12% body mass reduction in male *Egr1*cKO mice. Similarly, an approximately 14% reduction win body mass as observed for female *Egr1*cKO mice, which weighed on average 17.7 ± 0.68 g, while female *Egr1*^{f}Nes$^{cre–}$ mice weighed 20.7 ± 1.50 g on average. In the early weeks, some significant differences in weight were sporadically observed. This is likely due to technical variations being enlarged by the lightweight litter. Interestingly, compared with female *Egr1*^{wt}Nes^{cre+} mice, slightly decreased body weights were observed in the female *Egr1*^{f}Nes^{cre+} mice, with p values ≤ 0.05 at six and twelve weeks. This result suggests that the loss of *Egr1* might have a more noticeable impact on female mice than on male mice.

Sex Differences in Gene Expression Amplified in Nestin-Cre and *Egr1*cKO Mice

The growth differences observed in the Nestin-Cre and *Egr1*cKO mice inspired us to examine their pituitary gene expression profiles. RNA-seq analyses were performed in biological triplicates on adult pituitary tissues isolated from the eight groups of mice, with each sample taken at 12 weeks of age. A principal component analysis (PCA) indicated that, according to their gene expression patterns, the twenty-four samples could be segregated into two clusters, with females on the right side of the graph and males on the left (Figure 3.3A). Pearson correlation coefficients of the gene expressions across the samples further confirmed sex-specific clustering in general (Figure 3.3B). Intriguingly, strong correlations in the gene expressions in the pituitary tissues across all four genotypes were observed for males but not for females. More specifically, despite a strong correlation among the other three biological replicates, the gene expression profiles from the female *Egr1*^{f}Nes^{cre+} pituitary tissues showed a weak correlation (a Pearson's r of around 0.2–0.4) with those of the Nes$^{cre–}$ mice, but intermediate to strong correlations (Pearson's rs of approximately 0.6–0.8) with those of both male and female Nes^{cre+}

mice. This result suggests that gene expression differences resulting from different genotypes (Nes^{cre+} in particular) may prevail over sex differences.

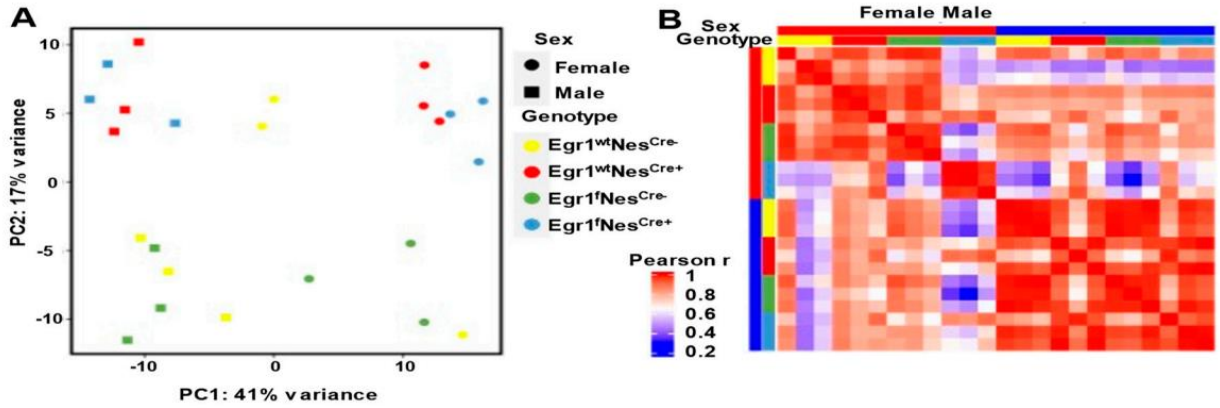

Figure 3.3. RNA-seq data clustering. **(A)** Principal component analysis (PCA) and **(B)** Pearson correlations of pituitary gland transcriptome data. RNA-seq analyses were performed in triplicates for each group, and all samples were from mice 12 weeks of age.

We thus performed pairwise comparisons for the four genotypes separately to identify the genes associated with sex-specific pituitary expression. For the wild-type mice (*Egr1*^{wt}Nes^{cre-}), a total of 217 genes (99 downregulated and 118 upregulated) were observed in the females (Figure 3.4A, Supplementary Tables S1–S4). The number of differentially expressed genes (DEGs) involved in sex-related differences increased to 481 in the *Egr1*^{f}Nes^{Cre-} mice, 1925 in the *Egr1*^{wt}Nes^{Cre+} mice, and 3822 in the *Egr1*^{f}Nes^{Cre+} mice. A large number of the DEGs identified in the two sexes were genotype-specific (Figure 3.4B, Supplementary Tables S1–S4). Only 24 upregulated genes in females and 20 upregulated genes in males were found to be shared by all four genotypes. These shared genes are predominantly from the sex chromosomes and include Ddx3y and Kdm5d on the Y chromosome and Xist and Kdm6a on the X chromosome. For each pairwise comparison, we calculated the percentage of DEGs that were identified in autosomes and sex chromosomes (Figure 3.4C). Compared with the wild-type controls, the percentage of DEGs in autosomes increased by approximately 3.5 and 4.0 times for the female and male *Egr1*^{f}Nes^{cre+} mice, respectively. For all four genotypes, the percentage of upregulated pituitary DEGs on the sex chromosomes in the males was approximately twice as high as that observed in the females. Altogether, the sex-related differences in pituitary gene expression were amplified in the *Egr1*^{f} and Nes^{cre+} mice and involved a thousand genes on autosomes.

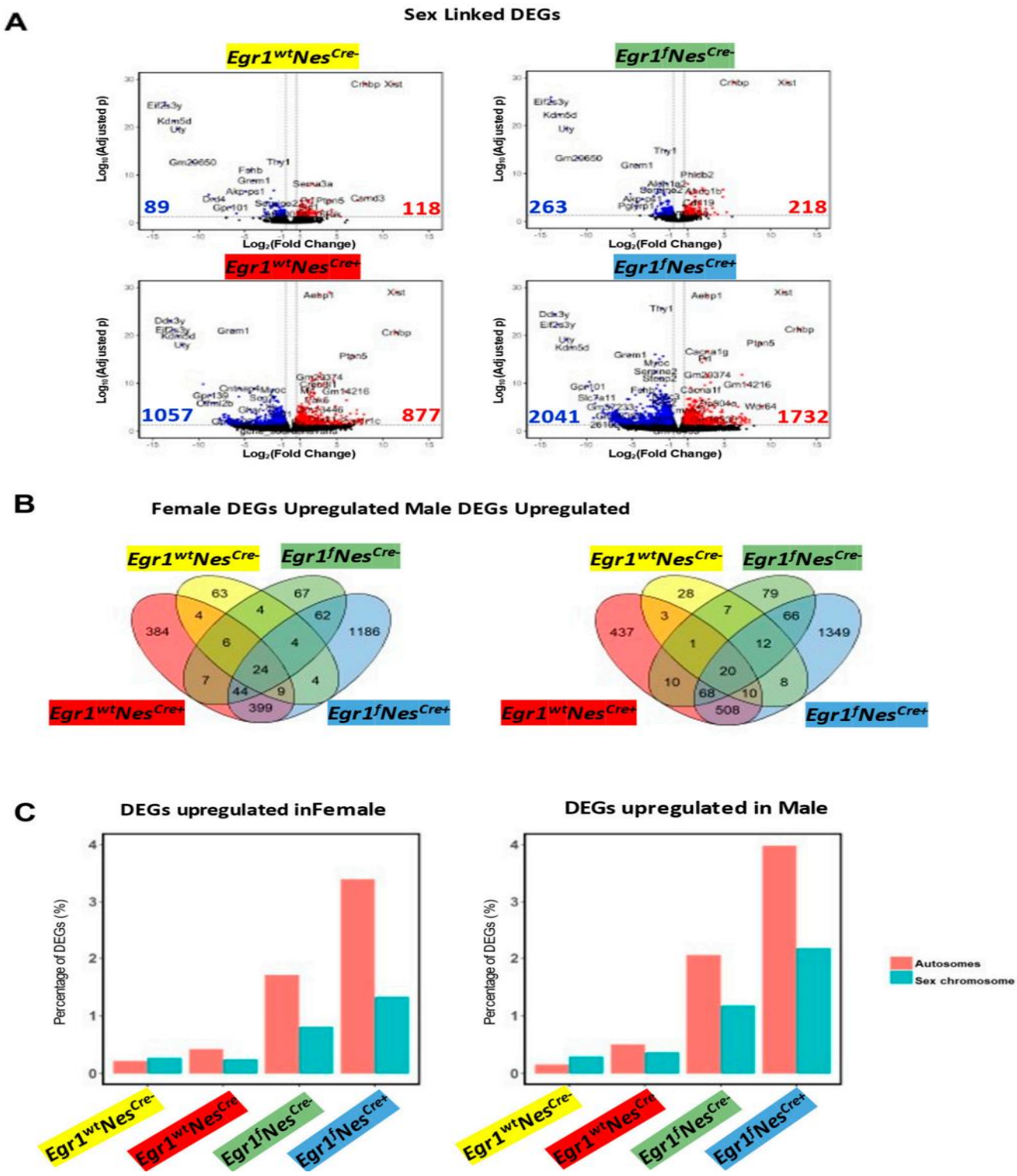

Figure 3.4. **Gene expression changes associated with sex difference**. **(A)** Volcano plot for pairwise analysis between two sexes for four genotypes. DEGs were defined as genes with FCs ≥ 1.5 and adjusted p values ≤ 0.05. Blue dots indicate downregulated DEGs in males. Red dots indicate upregulated DEGs in males. Grey dots indicate

no DEGs. (**B)** Venn diagram showing the overlapped DEGs identified in the pairwise comparisons. Corresponding genotypes are color-coded (blue for *Egr1*^{f}Nes$^{cre+,}$ green for *Egr1*^{f}Nes$^{cre–}$, red for *Egr1*^{wt}Nes^{cre+}, and yellow for *Egr1*^{wt}Nes$^{cre–}$). **(C)** Chromosome distribution of DEGs.

Aberrant Gene Expression Associated with Nestin-Cre and *Egr1*cKO Mice

The substantial sex-related differences in pituitary gene expression observed in the mice of four different genotypes suggest that the Nestin-Cre driver may have a distinct influence on the development of male and female pituitary tissues. To better understand the effect of the Nestin-Cre driver, we focused on the *Egr1*^{wt}Nes^{cre+} and *Egr1*^{wt}Nes$^{cre–}$ mice. We still kept the male and female RNA-seq data separate to perform pairwise comparisons (Figure 3.5A, Supplementary Tables S5 and S6). In the females, 668 genes were downregulated, and the Nestin-Cre driver upregulated 259 genes. Fewer DEGs were found in the males (159 downregulated genes and 90 upregulated genes). Only 38 DEGs upregulated in *Egr1*^{wt}Nes^{cre-} and 4 DEGs upregulated in *Egr1*^{wt}Nes^{cre+} pituitary glands were found to be shared by the male and female mice (Figure 3.5B). Significant differences in the enrichment of GO terms were observed between the female and male DEGs, and none of the GO terms were enriched for genes downregulated by the Nestin-Cre driver in the male pituitary, likely because only a small number of DEGs were detected (Figure 3.5C).

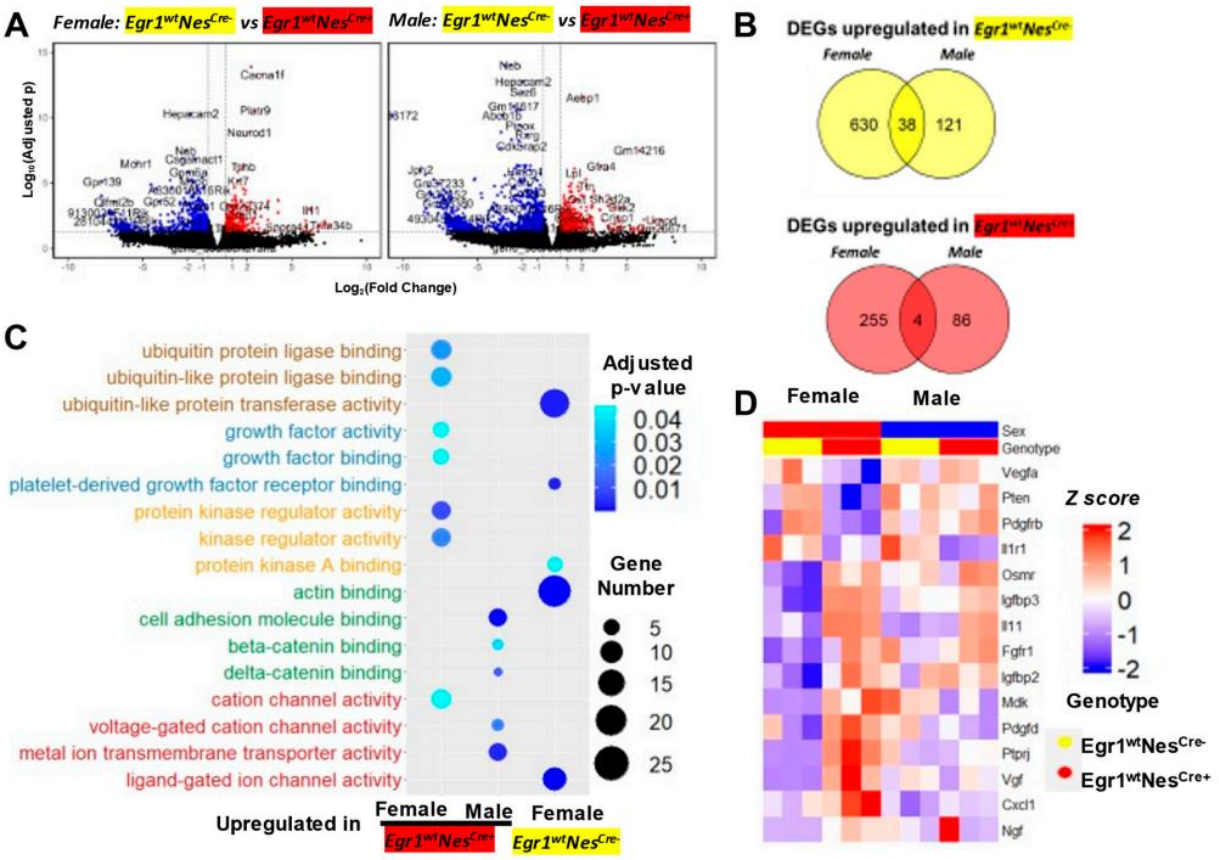

Figure 3.5. **Gene expression changes associated with the Nestin-Cre deriver**. **(A)** Volcano plots for pairwise comparisons between female (left) and male (right) *Egr1*^{wt}Nes^{cre+} and *Egr1*^{wt}Nes^{cre-} mice. **(B)** Venn diagram showing the overlapped DEGs identified in the pairwise comparisons for the two sexes. **(C)** GO analysis of identified DEGs. **(D)** Gene expression profiles of 15 DEGs were identified in the 3 GO categories associated with growth factors.

Five categories of GO terms in biological processes were found to be significantly enriched for DEGs associated with the Nestin-Cre driver. These GO terms are related to growth factors, ubiquitin protein ligase, protein kinase, cell adhesion molecules, and ion channel activity. Interestingly, the genes upregulated and downregulated by the Nestin-Cre driver in females were frequently found in similar GO categories. Although it remains unknown how these genes coordinate with each other in response to the Nestin-Cre driver, the identification of DEGs in the functional pathway related to growth factor may help explain the smaller stature of the Nestin-Cre mice. The expression patterns across 12 samples were examined for the 15 genes associated with the GO terms related to growth factors (Figure 3.5D). Except for interleukin 1 receptor type 1 (Il1r1), all of the genes were found to be Nestin-Cre-associated DEGs in females, but not in

males. Compared with the wild-type controls, the expression of the Il1r1 gene decreased with a fold change of 0.22 in the males and 0.39 in the females. Interleukin 1 (IL-1) stimulates the expression of various kinds of growth factors, including fibroblast growth factor-2 [26] and nerve growth factor [27]. However, the mice without the Il1r1 gene exhibited no overt phenotype but did exhibit altered innate immune and inflammatory responses [28]. Since the Nestin-Cre driver led to a large number of genes being differentially expressed in the pituitaries of the two sexes, we performed four kinds of pairwise comparisons to examine the influence of *Egr1*cKO. In the females, more DEGs (916 upregulated and 435 downregulated) were identified between the *Egr1*^{f}Nes^{cre+} and *Egr1*^{f}Nes^{cre-} mice than the number of DEGs (668 upregulated and 259 downregulated) identified between the *Egr1*^{wt}Nes^{cre+} and *Egr1*^{wt}Nes^{cre-} mice (Figure 3.6A). However, in males, an opposite trend in the number of DEGs was observed; more DEGs were identified for the Nestin-Cre driver alone (Figure 3.6B). Since a pairwise comparison is not sufficient to scrutinize the influence of conditional knockout of the *Egr1* gene, we adopted a mixed-gene model to analyze the pituitary gene expression profiles of the mice of all four genotypes (Supplementary Table S7). Only the Serpina3c gene was determined to be associated with *Egr1* loss in the males, while eleven such genes were identified in the females (Figure 3.6C). We further examined the expression profiles of the *Egr1*, growth hormone, and LH_{β} genes, and they were not differentially expressed across genotypes. Altogether, our results indicate that the conditional knockout of the *Egr1* gene has a more severe impact on pituitary gene expression in females than in males. In addition, in males, the influence of *Egr1*cKO on pituitary gene expression may be overridden by the Nestin-Cre driver.

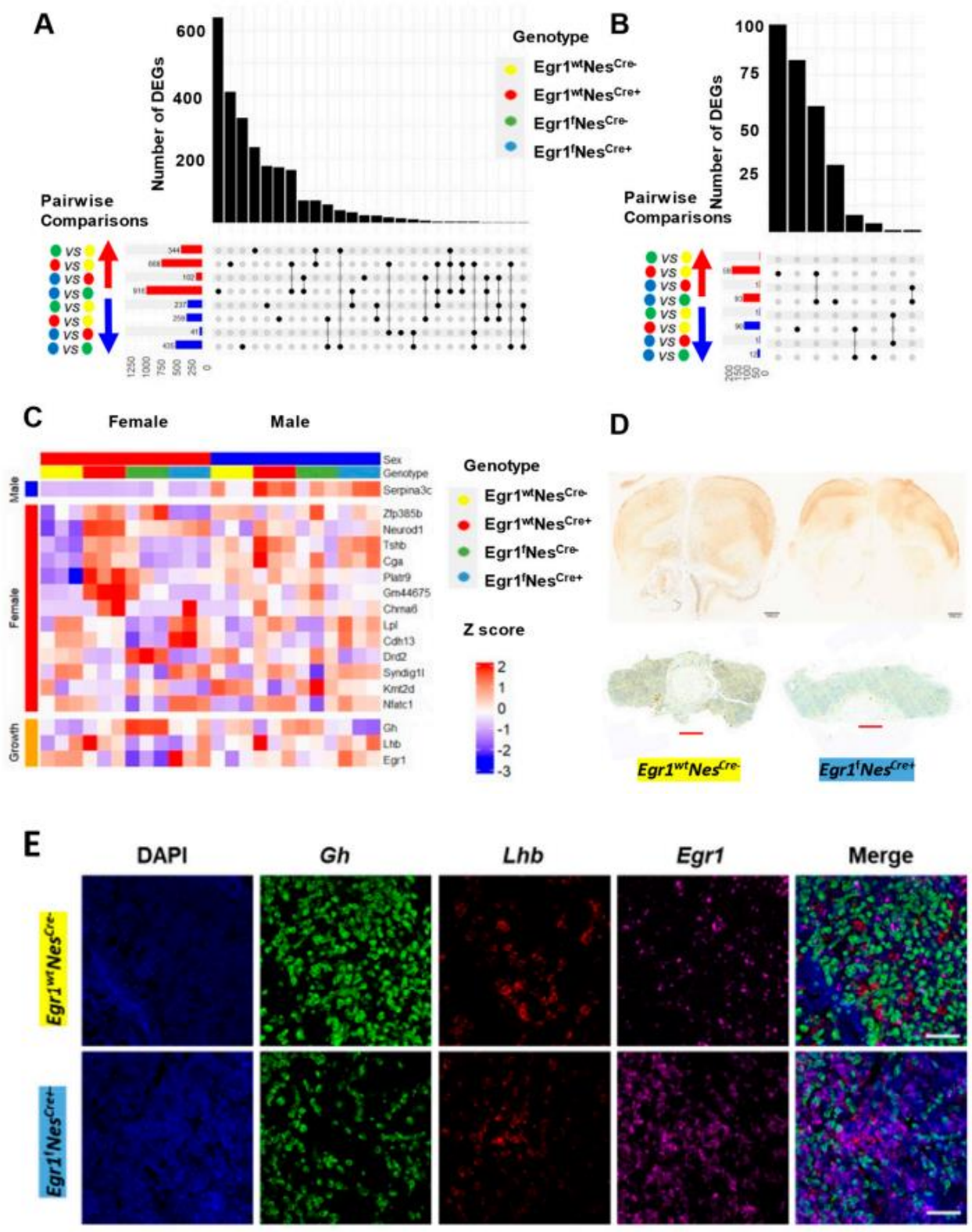

Figure 3.6. Gene expression changes associated with *Egr1*cKO. Bar-plots showing the number of intersecting DEGs identified in pairwise comparisons between **(A)** females and **(B)** males.

Horizontal bars on the bottom left side of each plot show the numbers of DEGs determined to be upregulated (red arrow) or downregulated (blue arrow). Different intersection combinations of DEGs identified for each pairwise comparison are represented by the dot-plot. The vertical bar-plots show the number of intersecting DEGs in the indicated combinations of pairwise comparisons. (C) Gene expression profiles of *Egr1*, growth hormone, and LH_β together with DEGs determined to be associated with *Egr1*cKO (1 DEG in males and 11 DEGs in females). (D) Immunohistochemistry staining of mouse brain in coronal view (scale bar = 2 mm) and pituitary tissues of *Egr1*cKO mouse and wild-type control. (scale bar = 400 μm). (E) RNAscope results for *Egr1*, growth hormone, and LH_β expression in the pituitary tissues of *Egr1*cKO and wild-type mice (scale bar = 50 μm). The experiments involving IHC and RNAscope were replicated using biological duplicates.

To validate the presence of EGR1 proteins, immunohistochemistry using an anti-Egr1 antibody was performed on the brain sections and pituitary tissues obtained from the adult *Egr1*^{f}Nes^{cre+} and *Egr1*^{wt}Nes$^{cre–}$ mice (Figure 3.6D). EGR1 proteins were widely distributed across the cerebrum and highly expressed in the hippocampus in the *Egr1*^{wt}Nes$^{cre–}$ mice, but they were substantially diminished in the brain sections derived from the *Egr1*^{f}Nes^{cre+} mice. However, EGR1 proteins were detected in the pituitary tissues of both the *Egr1*^{wt}Nes^{cre+} and *Egr1*^{f}Nes$^{cre–}$ mice. This result indicates that the Nestin-Cre driver removes the expression of the *Egr1* gene from neural lineage cells in the brain but fails to knock out the *Egr1* gene in pituitary cells. Lastly, we further confirmed the expression of the *Egr1*, growth hormone, and LH_β genes in the pituitary tissues using RNAscope (Figure 3.6E). For each probe, individual images of the anterior pituitary were taken along with a DAPI control (Figure 3.6E). Consistent with IHC staining (Figure 3.6D), *Egr1*, LH_β, and GH mRNAs were detected in the pituitary glands of both the *Egr1*^{f}Nes^{cre+} and *Egr1*^{wt}Nes$^{cre–}$ mice. Such findings were observed consistently in both the IHC and RNAscope experiments using biological duplicates.

3.6 Discussion

Nestin-Cre transgenic mice have been widely used to study gene functions in the central nervous system. In this study, we found that the Nestin-Cre driver is able to successfully knock out *Egr1* expression in a large number of brain cells but that it fails to remove *Egr1* from all three sections of the pituitary. Despite the high expression of Nestin previously found in the pituitary progenitor cells [25], a previous study did not find intensive expression of the Nestin-Cre transgene in the embryonic or adult pituitary [29]. Such inconsistency may be explained by the

fact that different reporter mice were used in these studies and by the unique characteristics of Nestin-Cre expression. During pituitary development, a high level of Nestin expression was observed in POU1F1 (POU domain class 1 transcription factor 1)-positive progenitor cells, but this was followed by a drastic reduction in the postnatal period [24]. The cells with Nestin expression in the postnatal period were terminally differentiated and located in the intermediate and posterior pituitary lobes. However, Nestin-Cre mediated recombination is extremely infrequent in early embryonic progenitor cells but can reach nearly 100% in neural and glial progenitor cells during perinatal development [30]. In this study, our results concerning *Egr1* mRNA and protein expression profiles have further confirmed the speculation that this specific strain of Nestin-Cre (B6.Cg-Tg (Nes-Cre)1Kln/J; Jackson Lab, #003771) may not be suitable for pituitary gene knockout. Despite this limitation, we made several interesting observations. Both *Egr1* loss and the Nestin-Cre driver have been reported to be associated with growth abnormalities in transgenic mice [17,23]. In this study, for mice of four genotypes related to *Egr1*cKO driven by Nestin-Cre, we provided growth curves to monitor their postnatal changes in detail. The differences in growth rate resulting from the Nestin-Cre driver alone became significant as young adulthood approached and were consistent after eight weeks. Compared with the female groups, the *Egr1*^{wt}Nes^{cre+} males with the Nestin-Cre driver showed more significant differences from the controls, but no additional weight loss was observed in the *Egr1*^{f}Nes^{cre+} males with the combination of Nestin-Cre and *Egr1* knockout in the neuronal lineage cells of the brain. However, *Egr1* knockout led to slightly decreased body weight in the *Egr1*^{f}Nes^{cre+} females compared with the *Egr1*^{wt}Nes^{cre+} Females. This sex-related difference was further manifested in the gene expression profiles. Considering the significant role of the pituitary in the regulation of growth and development, we explored whether and how its expression profile may be altered in response to the loss of *Egr1*. To our surprise, the *Egr1* gene was indeed removed from the neuronal lineage cells in the brain, but it was still expressed in the pituitary cells. This raises the possibility that the reductions in body size may have been due to the loss of *Egr1* in the neuronal lineage cells. Regardless, we determined in this study that this loss of *Egr1* expression resulted in substantial gene expression changes in the pituitary and affected the activities of growth factors. In our study, a total of 217 genes were determined to be differentially expressed in the pituitary tissues of male and female wild-type controls. This difference increased approximately two-fold in the *Egr1*^{f}Nes^{Cre-} mice, five-fold in the

Egr1^{wt}Nes^{Cre+} mice, and nineteen-fold in the *Egr1*^{f}Nes^{Cre+} mice. This result suggests that *Egr1*^{f} and Nes^{Cre+} take on distinct functions, but that together, they amplify their impact on the regulation of sex related pituitary gene expression. The presence of the Nestin-Cre driver alone resulted in 927 differentially expressed genes in the female pituitary, but only 249 genes were affected in the male pituitary tissue. A GO term analysis showed that the pathway associated with growth factor was enriched in the list of differentially expressed genes. This may help explain the growth changes observed in the Nestin-Cre mice. Using a mixed-gene model, we identified a small set of differentially expressed genes associated with the loss of *Egr1* in neuronal lineage cells, but the strong influence of the Nestin-Cre driver may undermine the impact of *Egr1* on pituitary gene expression. Future studies using different Cre lines are highly desired to further understand the roles of *Egr1* in pituitary functions.

3.7 Conclusion

Altogether, our results indicate that the Nestin-Cre driver was able to efficiently remove *Egr1* genes from neuronal lineage cells in the brain but not from pituitary cells. An RNA-seq analysis revealed that growth factor-related gene pathways were disrupted by the Nestin-Cre driver. In addition, sex differences in growth and gene expression were observed in the *Egr1*cKO and Nestin-Cre mice, and female mice were more sensitive to the presence of Nestin-Cre.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biology12070966/s1, Supplementary Table S1. Full list of DEG sex differences, female vs. male (*Egr*1^{wt}Nes$^{cre–}$); Supplementary Table S2. Full list of DEG sex differences, female vs. male (*Egr1*^{f}Nes$^{cre–}$); Supplementary Table S3. Full list of DEG sex differences, female vs. male (*Egr1*^{wt}Nes^{cre+}); Supplementary Table S4. Full list of DEG sex differences, female vs. male (*Egr1*^{f}Nes^{cre+}); Supplementary Table S5. Full list of DEG differences between female *Egr1*^{f}Nes^{cre+} and *Egr1*^{wt}Nes^{cre+}; Supplementary Table S6. Full list of DEG differences between male *Egr1*^{f}Nes^{cre+} and *Egr1*^{wt}Nes^{cre+}; Supplementary Table S7. Full list of DEGs derived from mixed-model analysis.

Author Contributions: Conceptualization, C.S. and H.X.; resources, C.S., X.X., M.L. and K.Z.; data curation, Y.Z. and Y.L.; formal analysis, Y.Z., Y.L., C.S. and H.X; writing—original draft

preparation, C.S., Y.L., X.X. and H.X.; writing—review and editing, C.S., Y.L., X.X. and H.X. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All procedures were performed in accordance with national and international guidelines and approved by the institutional board at Virginia Tech. This study was conducted in accordance with National Institute of Health guidelines.

Informed Consent Statement: Not applicable. Data Availability Statement: The RNA-seq datasets generated in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE108768.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 4 Conclusion and Future Directions

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4.1 Conclusion

Early growth response 1 (*Egr1*) is a transcription factor that is important for both learning and memory and pituitary function (Bliss et al., 2009; Duclot & Kabbaj, 2017). Nestin-Cre is a special system that removes specific genes from the central nervous system (Bernal & Arranz, 2018). The cross of *Egr1-floxp* and Nestin-Cre mice is one of the most advanced options to achieve *Egr1* deletion in the central nervous system.

In Chapter 2, results supported that the Nestin-Cre driver can efficiently remove *Egr1* genes in neuronal lineage cells in the hippocampus. RNA-seq analysis revealed that Egr1cKO disrupted Wnt signaling and axon guidance pathways. This pathway influences the extracellular matrix and provides evidence that *Egr1* influences the extracellular matrix and its role in memory (Rim et al., 2022). In addition, sex differences in behavior and gene expression were observed in the Egr1cKO mice, and female mice are more sensitive to the loss of *Egr1* gene.

In Chapter 3, results supported that the Nestin-Cre driver can efficiently remove *Egr1* genes in neuronal lineage cells in the brain but not pituitary cells. This is the same result shown in previous studies and was attributed to poor recombination of Nestin-Cre insertion into the adult pituitary. Previous studies have linked hypopituitarism to Nestin-Cre mice due to smaller growth size in strains used to form gene deletion study strains; however, no definitive links to growth through molecular pathways or physiological analysis (Galichet et al., 2010). The RNA-seq analysis revealed that growth factor binding and activity are disrupted by Nestin-Cre driver and are linked to females. In addition, sex differences in growth and gene expression were observed in the *Egr1*cKO and Nestin-Cre mice, and female mice are more sensitive to the presence of Nestin-Cre.

Finally, the data collected in Chapter 2 and Chapter 3 resulted in two key findings. The first is that females are more affected by removal of *Egr1* in the body than males; no definitive reason has been identified. Sex is an important variable and should be included in any future study to evaluate this transcription factor. The second finding is that when approaching gene deletion studies for function with Nestin-Cre, all parts of the strategies to employ the desired targets should each have control to appropriately interrupt the data found (Giusti et al., 2014).

4.2 Future Directions

In Chapter 2, sex differences and some sex-linked behavioral differences were identified in the hippocampus RNAseq data. The more profound finding was that neuronal *Egr1* did not seem to influence behavior as previously thought. To make this research even more meaningful, it would be recommended that the same strain of *Egr1*cKO Nestin-Cre be evaluated in separate behavioral paradigms that appropriately address the hippocampal function, such as the water maze (Othman et al., 2022). This will allow for confirmation of testing results in a different setting addressing the same areas of brain function. Once confirmed for repeated results in different tests for hippocampal function, other brain regions, such as the amygdala, can be used to check for brain region-specific function with *Egr1*cKO.

In Chapter 3, the pituitary from *Egr1cKO* mice was found to have *Egr1* expression different from what observed in the brain. However, it still had many gene expression differences found in RNA-seq analysis. Since the breeding scheme used to create this particular strain of mice was heterozygous Nestin-Cre, there could be two different directions for future studies. One direction would be to use homozygous Nestin-Cre mice, which should give entirely new RNA-seq results and could be used in comparison with the current RNA-seq data. The other genomic direction would be to employ single cell technology to evaluate all cellular data in the pituitary (Ho et al., 2020). Both options could deliver a more precise information to understand the growth pathways. Other data were collected during this study that identified weight differences in the liver, testis, brain, kidney, and spleen of the males but not in the females, which differs from the overall impression in Chapter 2 and Chapter 3 and warrants its own study. Exploring this sex difference in growth could help us understand different developmental processes linked to pituitary affected growth pathways and their primary function in homeostasis within the different sexes.

One proposed difference in the cause of the behavioral and gene expression changes is that *Egr1* has other compensatory mechanisms in the rest of the body. In a previous study looking at postmortem samples from patients diagnosed with schizophrenia, it was found that EGR1 had lower expression in the frontal lobes. Still, these patients had higher EGR1 responses in the peripheral blood samples (Duclot & Kabbaj, 2017). This interesting finding from patients with a mental health disorder and EGR1 expression can be the stepping stone to understanding EGR1 expression, either increased or decreased and compensatory mechanisms in the body.

Mice from the Egr1cKO strain driven by Nestin-Cre could be used to measure peripheral blood sample EGR1 expression and compare expression to the nervous system to confirm higher expression of EGR1 in the peripheral blood since *Egr1* was primarily removed from central nervous system but rather lymphatic system.

Egr1 is a gene that has shown its importance in learning, memory, and pituitary function. Our two studies have resulted in new information regarding *Egr1* role in sex specific growth patterns and loss of *Egr1* in neuronal lineages for learning and memory. These important physiological processes now have a greater understanding and many future directions to expand upon for a greater scientific impact.

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