DISCOVERING THE EFFECTS OF INTRINSIC AND EXTRINSIC SIGNALS ON THE DEVELOPING PITUITARY GLAND

BY

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DISSERTATION

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ABSTRACT

The pituitary gland acts as a “master gland” of the endocrine system and mediates multiple physiological processes. Dysfunctions in pituitary development can lead to hypopituitarism, a deficiency in pituitary hormone secretion that causes disrupted endocrine functions. Intrinsic signals including sequential expression of transcription factors and activation of multiple signaling pathways, as well as extrinsic signals including hormone signals from other endocrine glands and exposure to endocrine disrupting chemicals are two regulators for pituitary development. Understanding of the mechanisms by which the intrinsic and extrinsic signals regulate the pituitary development is necessary for us to understand the proper functioning of the gland, and thus help us to develop treatments for hypopituitarism.

The NOTCH signaling pathway has been known to be an intrinsic regulator of pituitary development and our lab’s previous studies have indicated that it’s necessary for pituitary stem cell renewal, maintenance, and differentiation. In this study, we identified a novel pituitary stem cell marker- glycoprotein hormone subunit alpha 2 (GPHA2), which we found to be regulated by NOTCH2, and we hypothesized it functions as a paracrine factor secreted by stem cells to regulate neighboring cells’ maintenance. We applied techniques including single cell RNA sequencing analysis, quantitative real-time PCR, *in situ* hybridization, immunohistochemistry, western blot, and pituitary explant/cell cultures. We identified GPHA2 signals through the thyroid stimulating hormone receptor (TSHR) signaling pathway in the pituitary and provided a target receptor for future investigations on GPHA2’s functions in the pituitary.

Di(2-ethylhexyl) phthalate (DEHP) is an endocrine disrupting chemical (EDC) that’s known to interfere with reproductive functions. In this study, we investigated DEHP’s impacts as an extrinsic signal on the developing pituitary gland. We hypothesized DEHP activates aryl hydrocarbon receptor (AhR) signaling pathway in the pituitary and impacts gonadotroph functions. We performed *in vivo* dosing and *in vitro* pituitary explant culture, with analysis techniques including quantitative real-time PCR and dual-luciferase reporter assay. We reported DEHP (and its primary metabolite mono(2-ethylhexyl) phthalate, MEHP) to have both systemic and direct actions on the pituitary gland. Specifically, we found MEHP activates AhR signaling directly on the pituitary and caused a decrease in gonadotropin expression. Although we found no direct association between AhR activation and the reduction in gonadotropin expression, our study provided a direction for future studies in terms understanding the regulation of gonadotroph development and functions, possibly in relation to AhR signaling pathway in the pituitary.

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CHAPTER 1: INTRODUCTION

Abstract

The pituitary gland is crucial for regulating the endocrine control of many physiological systems and its functions could be impaired by exposure to endocrine disrupting chemicals (EDCs). Humans and animals are exposed to EDCs throughout life, but exposure during critical periods when the organs are developing could have more damaging consequences. In this review, we summarized the development of pituitary gland regulated by intrinsic and extrinsic signals, and the effects of *in vivo* EDC exposure during development on the pituitary. These include changes in pituitary hormone, mRNA and protein expression levels, as well as pituitary cell number and properties. Each hormone-producing cell type and pituitary stem/progenitor cells was reviewed, and we pointed out the current gaps in knowledge and suggested future directions in terms of understanding effects of intrinsic and extrinsic signals on pituitary gland development.

Introduction

The pituitary gland is the “master gland” of endocrine system, and it is responsible for regulating various physiological processes including body growth, stress response, energy metabolism, pigmentation, reproduction, lactation, and water balance. The pituitary gland regulates these complicated processes by taking signals from the body and executing its functions via a group of cells that make up the gland: somatotrophs- produce growth hormone (GH), corticotrophs- produce adrenocorticotropic hormone (ACTH), thyrotrophs- produce thyroid stimulating hormone (TSH), melanotrophs- produce melanocyte-stimulating hormone (αMSH), gonadotrophs- produce gonadotropin: luteinizing hormone (LH) and follicle-stimulating hormone (FSH), lactotrophs- produce prolactin (PRL), and posterior pituitary cells- produce oxytocin and vasopressin (ADH) (Drouin 2017). Besides, there are pituitary stem/progenitor cells responsible for populating the gland with these hormone cells (Nantie et al. 2014; Edwards and Raetzman

2018) and folliculostellate cells responsible for cell networking and intrapituitary interactions (le Tissier and Mollard 2021).

Dysfunctions in the pituitary could result in impacts across different organs and systems in the body. Specifically, congenital pituitary hormone deficiencies (hypopituitarism) such as GH, TSH, ACTH, LH and FSH deficiency (Drouin 2017) lead to growth retardation (Tritos and Klibanski 2016) and reproductive disorders (de Angelis et al. 2017; Mikhael et al. 2019), among other problems. Genetic causes of combined pituitary hormone deficiency (CPHD, also known as panhypopituitarism), which is characterized by combined shortage of GH and at least one other pituitary hormone, include mutations in *Prop1*, *Pou1f1*, *Hesx1*, *Lhx3*, and *Lhx4* (Castinetti et al. 2016; Fang et al. 2016; Gregory and Dattani 2020). Despite intensive studies to identify mutations that cause hypopituitarism, only 16% of patients with hypopituitarism are diagnosed with known genetic abnormalities (Bosch i Ara et al. 2021). This challenge in determining causes of hypopituitarism indicates environmental factors may also contribute to the dysfunction alongside genetic factors.

Environmental factors that could cause pituitary dysfunctions include traumatic injuries, infections, and chemical exposures. Among all the environmental factors associated with pituitary dysfunction, the effects of endocrine-disrupting chemicals (EDCs) are attracting increasing research interest as humans are constantly exposed to them. Commonly known types of EDCs and their uses are industrial EDCs including dioxins, brominated flame retardants, tributyltin (TBT), and polychlorinated biphenyls (PCBs); pesticides including dichlorodiphenyltrichloroethane (DDT), neonicotinoids, and endosulfan; plasticizers including phthalates and bisphenol A (BPA); pharmaceutical EDCs including parabens and triclosan; and heavy metals including arsenic and mercury (Lauretta et al. 2019; Yilmaz et al. 2020). EDC exposures can have multiple mechanisms of action including activating or inhibiting hormone receptors, activation of kinase signaling pathways, interference with hormone feedback regulation, disruptions in hormone production, transportation, and metabolism (Yilmaz et al. 2019), and epigenetic regulation (Alavian-Ghavanini et al. 2017; Wen et al. 2020).

Although humans and animals are constantly exposed to EDCs, some periods of exposure are considered critical windows during which exposure can cause more serious consequences. Critical windows include gestation, the neonatal period, and puberty (Palanza et al. 2016; Kumar et al. 2020) as those are developmental periods when the pituitary goes through intensive tissue proliferation, differentiation, and maturation. Many of the impacts of developmental exposure to EDCs could last into adult life (Horzmann et al. 2021) and even be passed on to the next generations (Brehm et al. 2018; Drobná et al. 2018; Wen et al. 2020). In this review, we will go through the development and regulation of the pituitary gland and how exposure to EDCs can affect the developing pituitary. We will discuss each distinct hormone-producing cell type in the pituitary and how exposure to EDCs could affect them during pituitary development (Table 1.1). We will also consider what is known about intrinsic signals regulating pituitary development, focusing on the NOTCH pathway.

Intrinsic regulation of pituitary gland development

The anterior and intermediate pituitary lobes originate from Rathke's pouch, an invagination from oral ectoderm. The posterior pituitary originates from an evagination from neural ectoderm, derived from the ventral diencephalon (Daly and Camper 2020) (Figure 1.1 A). Together they form the pituitary gland starting from gestational week 5 in humans and embryonic day 10.5 (e10.5) in mice (Alatzoglou et al. 2020).

The development of hormone-producing cells in the anterior and intermediate pituitary is regulated by various transcription factors (TFs) which sequentially activate different signaling pathways (Figure 1.1 B). The earliest TFs activated are Pituitary Homeobox 1 (*Pitx1*) and *Pitx2*, followed by expression of LIM Homeodomain Protein 3 (*Lhx3*) and *Lhx4*. Different hormone producing cells have unique TFs including corticotrophs marked by T-Box Transcription Factor

19 (*Tbx19*), melanotrophs marked by *Tbx19* and Paired Box 7 (*Pax7*), gonadotrophs marked by Nuclear Receptor Subfamily 5 Group A Member 1 (*Nr5a1*), and POU Class 1 Homeobox 1 (*Pou1f1*) positive cells which then differentiate into somatotrophs, lactotrophs and thyrotrophs (Alatzoglou et al. 2020). Numerous TFs and signaling pathways are involved in maintenance and cell fate decision of stem/progenitor cells in the pituitary gland. The major pituitary stem cell specific TFs include SRY-Box Transcription Factor 2 (*Sox2*) and *Sox9* (Daly and Camper 2020). The population of pituitary SOX2/SOX9+ cells decreases as differentiation proceeds; however, they mostly reside along the cleft in the adult pituitary and play a limited role in maintaining organ homeostasis (Haston et al. 2018).

Major signaling pathways involved in pituitary stem/progenitor cell regulation include Sonic Hedgehog (SHH), wingless (WNT), and NOTCH signaling. Specifically, the NOTCH signaling pathway is a highly conserved signaling pathway present in multiple organs including the pituitary gland. It's active in the early stage of embryonic development, and it's maintained in stem cells throughout the life of the organism. The NOTCH signaling pathway is involved in processes crucial to development, including regulation of stem cell renewal, maintenance, and differentiation. The activation of NOTCH signaling requires contact between NOTCH receptors and cell surface-expressed NOTCH ligands on adjacent cells, followed by cleavage of the receptor and release of NOTCH intracellular domain (NICD) into cytoplasm. NICD translocates into the nucleus and binds with transcription factor Recombination Signal Binding Protein For Immunoglobulin Kappa J (RBPJ), then the complex recruits coactivators/corepressors and eventually regulates the transcriptional activity of target genes including *Hes1* and *Hey1*. (Zhou et al. 2022).

In the pituitary gland, expression of *Notch2* and *Notch3* along with their ligands and effectors *Hes1* and *Hey1* can be detected during early development in Rathke's pouch (Raetzman et al. 2004; Zhu et al. 2006; Raetzman et al. 2007; Monahan et al. 2009), and the expression is maintained during postnatal development (Nantie et al. 2014; Zhu et al. 2015). NOTCH signaling is necessary for pituitary stem cell proliferation and maintenance as loss of either effector gene *Hes1* or the receptor *Notch2* leads to decreased pituitary volume, driven by decreased cell proliferation and increased cell cycle exit (Monahan et al. 2009; Nantie et al. 2014). Besides, NOTCH signaling is also essential for stem cell differentiation and lineage specification in the pituitary. NOTCH has been reported to block *Pomc* lineage (corticotroph and melanotroph) differentiation. NICD expression inhibited *Pomc* lineage differentiation (Goldberg et al. 2011) and induced them to regress toward a progenitor-like state (L. Cheung et al. 2018), and inhibition of NOTCH signaling led to increased *Pomc* mRNA levels (Nantie et al. 2014). On the other hand, NOTCH signaling is required for commitment of *Pit1* lineage as both knockout of *Rbpj* and *Notch2* in the pituitary caused decreased thyrotroph and somatotroph populations (Zhu et al. 2006; Nantie et al. 2014). In terms of mechanisms by which NOTCH functions in the pituitary, *Notch2* is thought to be regulated by *Prop1* (Raetzman et al. 2004) and reciprocally, *Prop1*'s expression was decreased in *Notch2*- or *Rbpj*- conditional knockout pituitaries (Zhu et al. 2006; Nantie et al. 2014). However, detailed mechanisms for the relationship and regulation between *Prop1* and *Notch2* still remain unclear. Besides, NOTCH signaling in relation to other TFs, its downstream targets specifically in the pituitary and their mechanisms of action are still in need for future investigation.

In addition to major signaling pathways, many other signaling pathways include mitogen-activated protein kinase (MAPK), RET/PIT1/p14ARF/p53 and PI3K/Akt pathways are also involved in regulation of pituitary development (Haston et al. 2018; Roof and Gutierrez-Hartmann 2018). Overall, the intrinsic regulation of pituitary gland development is delicate and complicated. The regulation could be impacted by many factors including exposure to EDCs, and studies have already reported EDC's effects on TF expression as well as alterations in signaling pathways in the pituitary (Brannick et al. 2012; Hannon et al. 2014).

Extrinsic regulation of pituitary gland development

The key functions of the pituitary gland are also regulated by hypothalamic signals, and any developmental changes in the number, neuropeptide expression or function of the neurons that contain these neuropeptides could impact pituitary function. Importantly, developmental EDC exposure is known to impact hypothalamic neuron populations and/or signaling networks (Graceli et al. 2020; Lopez-Rodriguez et al. 2021; Patisaul 2021). Other than regulating the secretion of pituitary hormones through hypothalamic-pituitary axis, hypothalamic signals can also impact the development of the pituitary gland. For example, in addition to stimulating the pituitary's release of GH, growth hormone releasing hormone (GHRH) is also essential for postnatal expansion of the somatotroph lineage specifically (Lin et al. 1993). For thyrotrophs, thyrotropin-releasing hormone (TRH) is required for their postnatal maintenance, but is not necessary for embryonic differentiation (Shibusawa et al. 2000). Interestingly, TRH can also induce the differentiation of gonadotrophs and lactotrophs, but whether the induction is direct is unknown (Heritier and Dubois 1993). Besides, gonadotropin releasing hormone (GnRH) has been found to induce the differentiation of gonadotrophs and thyrotrophs (Héritier and Dubois 1994).

The pituitary gland can also be regulated by other pituitary-expressed hormone receptors that take feedback signals from downstream endocrine organs of the hypothalamic-pituitary axis such as estrogen receptor (ER) (Arao et al. 2019), androgen receptor (AR) (Ryan et al. 2021) and thyroid hormone receptor (THR) (Ortiga-Carvalho et al. 2014). Disruptions in hormone signaling at the level of pituitary can cause impaired pituitary functions in mouse models. For example, ERα in the pituitary is found to be necessary for fertility in females as pituitary-specific ERα knockout (KO) mice presented with irregular estrous cycles and infertility (Gieske et al. 2008). Additionally, exogenous estradiol administration postnatally regulated gonadotroph gene expression (Eckstrum et al. 2016). Androgen signaling at the pituitary is crucial for repression of prolactin secretion and lactotroph development in males as pituitary-specific AR KO male mice showed hyperprolactinemia and female-like lactotroph development and distribution (O'Hara et al. 2021). Except for KO models, androgen is also known to regulate gonadotropin expression (Kreisman, Song, Yip, B. v. Natale, et al. 2017) and gonadotropin-releasing hormone receptor (*Gnrhr*) in gonadotrophs (Ryan et al. 2021). As for thyroid hormone, it is essential for maintaining the population of somatotrophs and lactotrophs in the mouse and chicken (Stahl et al. 1999; Liu and Porter 2004), and a congenital hypothyroidism mouse model exhibited increased numbers of thyrotrophs (Stahl et al. 1999). Importantly, hormone signaling at the pituitary could be affected by endocrine disruptions, including direct effects at the pituitary and through circulating hormone level changes, and thus impact pituitary development.

Given the complicated regulation of pituitary development by different intrinsic and extrinsic factors, disruptions in any of those mechanisms above could ultimately impact pituitary function. EDCs could potentially impact both extrinsic and intrinsic regulation of pituitary development. However, most current studies concerning EDC's effects on developing pituitary only focused on changes in circulating pituitary hormone levels later in life. Direct impacts of EDCs on the pituitary including changes in pituitary gene and protein expression during development, pituitary progenitor properties, as well as alterations in TF regulation and signaling pathways remain understudied. This review will detail the known effects of developmental EDC exposure on the pituitary and point out areas for further study.

Effects of developmental EDC exposure on gonadotrophs

Among all the endocrine functions the pituitary gland regulates, dysregulation in reproductive functions, which is mediated through gonadotrophs, is the most well-studied target of developmental EDC exposure. Effects of EDCs on gonadotroph functions could be reflected through different aspects: changes in serum gonadotropin hormone- LH and FSH levels, in gonadotropin mRNA and/or protein levels, in *Gnrhr* levels, and in gonadotroph cell numbers. Alterations in gonadotroph functions caused by EDC exposure can be observed in various animal models and sometimes the effects could be diverse depending on the types of EDCs, exposure windows, exposure dosage, sex differences, and timepoints of inspection.

Estrogenic and antiandrogenic EDCs like bisphenol A (BPA) and di(2- ethylhexyl) phthalate (DEHP) can often induce serum level of gonadotropins as well as inducing the mRNA or protein levels. For example, prenatal and lactational exposure to BPA (3 µg/kg/day) increased serum LH levels in female rats at postnatal day 30 (P30) (Gámez et al. 2015). Interestingly, different BPA exposure levels seem to have opposite effects at a younger age: female mice exposed to BPA from embryonic day 10.5 (e10.5) until e18.5 were found to have increased *Lhb*, *Fshb* and *Gnrhr* mRNA after being exposed to 0.5 µg/kg/day BPA at P1, but mRNA levels of those three genes were decreased in mice exposed to 50 µg/kg/day of BPA (Brannick et al. 2012). Prenatal and lactational exposure to DEHP caused an increase in *Lhb* mRNA in female mice (0.05, 5 mg/kg/day), as well as increase in *Lhb* and *Fshb* mRNA in males (5 mg/kg/day) at P21 (Pocar et al. 2012). Impacts of phthalates and BPA can be influenced by sex differences and various timepoints of inspection. In female quails exposed to DEHP (500, 1000 mg/kg) from P15 to P60 (a period before sex maturation), serum LH levels were increased at P60, however, FSH levels were decreased (Li et al. 2020). In rats, prenatal exposure to DBP (100 and 500 mg/kg/day) from e12 to e21 was reported to decrease only *Lhb* mRNA levels at P4, 7, 14 and 21, while 100 mg/kg/day DBP dosage increased the *Lhb* level at P28 and P56 (Chen et al. 2017). Besides, prenatal exposure to either BPA (25 µg/kg) or DEHP (50 µg/kg) in male rats could cause increases in LHβ and FSHβ protein levels at P35, but decreases in both proteins at P90 (Abdel-Maksoud et al. 2015).

In terms of understanding a mechanism for phthalate's disruption of gonadotropin expression, it is known that aryl hydrocarbon receptor (AhR) signaling impacts gonadotropin synthesis (see below) (Hattori et al. 2018), although the involvement of AhR in DEHP mediated gonadotropin changes is still under investigation. Prenatal exposure to the dioxin 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (1 µg/kg), a potent AhR ligand, at e15 decreased serum LH and FSH levels as well as decreased *Lhb* and *Fshb* mRNA levels in rat fetuses from e18 to P0. Mechanistically, TCDD induced increases in histone deacetylase levels and this correlated with a decrease in acetylated histone recruitment to *Lhb*'s promoter region. (Takeda et al. 2012).

Other than TCDD, early-life PCB exposure is correlated with reduced serum LH levels in prepubertal boys (Grandjean et al. 2012) and LH and FSH levels in male rats (PCB169, 30 μg/kg/day) (Yamamoto et al. 2005). Interestingly, female lambs were found to have increased gonadotropin releasing hormone (GnRH)-induced LH and FSH levels after prenatal and lactational exposure to PCB (PCB153, 49 μg/bw/day) (Kraugerud et al. 2012). Prenatal exposure to hydraulic fracturing chemicals (unconventional oil and natural gas (UOG) mixture at 3, 30, 3000 μg/kg/day) in female mice reduces serum LH and FSH levels at P85, which is a long-term effect of prenatal exposure (Kassotis et al. 2016). Finally, prenatal or early-life postnatal exposure to other EDCs such as pyrethroid insecticide cypermethrin (0.5, 5, 50 μg/kg), microcystin-LR (MC-LR, 30 μg/L), hexavalent chromium (100, 200 ppm) and TBT (10 mg/kg) have also been found to increase LH and/or FSH hormone levels and mRNA levels in both sexes in rats or zebrafish (Kariyazono et al. 2015; Hou et al. 2016; Ye et al. 2017; Shobana et al. 2020).

Developmental exposure to EDCs also affects gonadotroph cell numbers and properties. Prenatal exposure to low-dose BPA (0.5 μg/kg/day) increased gonadotroph cell number as well as pituitary proliferation in female mice at P1 (Brannick et al. 2012). Sheep prenatally exposed to sewage sludge had decreased numbers of LHβ- and ERα- positive gonadotrophs in both sexes at e110 (Bellingham et al. 2009). Other EDCs like di-n-butyl phthalate (DBP) and methoxychlor (MXC) have more complicated impacts on gonadotroph cell number: late embryonic through lactational exposure to DBP (10000 ppm) decreased FSH-positive cells, but increased LH-positive cells in both males and females at P21, while both sexes had increased FSH-positive cells at postnatal week 11 (PNW11) (Lee et al. 2004). Exposure to MXC (1200 ppm) during a similar period decreased LH- and FSH-positive cells in males, but only decreased LH-positive cells in females at PNW3. However, FSH-positive cells were increased in females at PNW11 (Masutomi et al. 2004).

In summary, examining the effects of various EDCs on reproductive functions, BPA and phthalates are two of the most well-studied types. As for their impacts on gonadotroph functions, specifically after developmental exposure, they were able to increase gonadotropin hormone and mRNA levels in both sexes at multiple ages in most cases. These relatively consistent outcomes indicate that the most likely mechanisms of their actions may be to interfere with the regulation of the hypothalamic-pituitary-gonadal (HPG) axis. These EDCs could directly impact gonadal steroidogenesis or block the negative feedback from estrogens or androgens on the pituitary and/or hypothalamus, thus making the pituitary increase the production of gonadotropins. However, BPA and phthalates, as well as other types of EDCs mentioned in this section, can have multiple modes of action. This is reflected in the fact that differing results were seen depending on sex, exposure window, dosage, and time of inspection. More studies need to be done to understand the mechanisms regulating how gonadotrophs in different conditions respond to developmental EDC exposure. In addition, although numerous studies reported changes in gonadotroph functions in response to developmental EDC exposure, most of them only focused on gonadotropin hormone and/or mRNA levels changes. Future studies are needed to better understand the direct impacts of developmental exposure to EDCs on gonadotrophs. These studies should examine proliferation and maintenance of gonadotrophs, alterations in TFs regulating gonadotrophs, like NR5A1 and FOXL2, changes in hormone receptors on gonadotrophs, and signaling pathways regulating the expression of gonadotropins like activins and inhibins.

Effects of developmental EDC exposure on lactotrophs

Like gonadatrophs, lactotrophs are another common target of developmental EDC exposure that is related to disruptions in reproductive functions, especially lactation. Changes in serum prolactin (PRL) levels, pituitary *Prl* mRNA levels, and lactotroph numbers are reported to occur with EDC exposure.

Studies in rodents revealed prenatal exposure to hexavalent chromium (100, 200 ppm) and hydraulic fracturing chemicals (UOG mixture at 3, 30, 300, 3000 μg/kg/day) decreased serum PRL levels in males at P30 (Shobana et al. 2020) and females at P85 (Kassotis et al. 2016), respectively. Other EDCs caused the opposite effect on PRL levels in multiple animal species: prenatal and lactational exposure to glyphosate-based herbicide (350 mg/kg/day) increased PRL levels in adult male rats (Gomez et al. 2019). As for females, exposure to DEHP (1000 mg/kg) from P15 to P60 increased PRL levels in quails (Li et al. 2020). BPA and phthalates are the most well-studied EDCs in terms of lactotroph function disruptions. In rats, prenatal exposure to BPA (250 μg/kg) increased PRL levels in males at P30 (Ramos et al. 2003), and similar effects of BPA (0.03, 3 ppm or 100, 300 mg/kg/day) were observed in mice and female rats with a longer exposure time (prenatal and postnatal exposure) (Kendig et al. 2012; Delclos et al. 2014). Researchers have also investigated the effects of exposure to mixtures of EDCs. Prenatal and perinatal exposure to an anti-androgenic mix (AAmix450) containing different types of phthalates and pesticides decreased PRL levels in female rats at P22 (Johansson et al. 2016).

Prenatal exposure to EDCs also changed the population and properties of lactotrophs in rats. Late embryonic and lactational exposure to DBP (10000 ppm) decreased the number of lactotrophs in both males and females at P21 (Lee et al. 2004). Interestingly, prenatal and lactational exposure to DEHP (200 μg/kg/day) increased the lactotroph population in adult females at P75 (Pérez et al. 2020). The reason for this opposite effect on lactotroph population caused by different types of phthalates is unknown and possible explanations include differences in phthalates (DBP vs. DEHP), different dosages (10000 ppm vs. 200 μg/kg/day), various developmental stages at the time of inspection (P21 vs. P75) and different exposure windows (e15 - P21 vs. e0 - P21). Late embryonic and perinatal exposure to MXC (1200 ppm) caused more complicated effects on lactotroph populations between different sexes at different ages: MXC exposure decreased lactotroph cell number at PNW3 in males but increased lactotroph cell number at PNW11 in females (Masutomi et al. 2004). In addition to increasing lactotroph number, DEHP (200 μg/kg/day) exposure affected the properties of lactotrophs as decreased proportions of ERα- and ERβ- positive lactotrophs were found in adult females (Pérez et al. 2020).

As the most well studied EDCs, BPA and phthalates generally increase PRL levels and/or the lactotroph population in developmentally exposed animals. These consistent outcomes may be explained by their estrogen-like properties, as estradiol is known to be able to induce lactotroph proliferation and PRL secretion (Zárate and Seilicovich 2010). However, the mechanisms for the induction are still unclear. Since PRL secretion from lactotrophs is generally inhibited by dopamine secreted from the hypothalamus (Dobolyi et al. 2020), it is possible that changes in lactotrophs' response to dopamine, such as changes in dopamine receptor expression and downstream signaling cascades, caused by EDC exposure could be part of the mechanism. The downregulation of estrogen receptors by DEHP in lactotrophs (Pérez et al. 2020) also indicates that DEHP may interfere with estrogen signaling pathways, and future studies are needed for understanding the detailed mechanisms. Given the important role lactotrophs play in regulating reproductive functions, more research is needed to better understand the mechanisms of direct impacts of developmental EDC exposure on lactotrophs. Specifically, since the development of lactotrophs is sex-dependent and they've been known to be sensitive to estrogen and androgen regulation (Ishida et al. 2007; O'Hara et al. 2021), future studies should also consider the EDCs' impacts on sex steroid hormone levels and receptor signaling in relation to lactotroph development and functions in different sexes. Another characteristic about lactotrophs is the fact that prolactinomas are the most prevalent form of pituitary adenoma (Pekic et al. 2021). Considering the constant exposure to EDCs in human lives, the question as to whether there's an association between developmental EDC exposure and prolactinoma formation is worth investigation. Specifically, future studies might examine the correlation between EDC levels and prolactinoma cases, and the possible impacts of developmental EDC exposure on lactotroph proliferation both acutely and later in life in response to additional environmental exposures.

Effects of developmental EDC exposure on thyrotrophs

Because of the requirement for congenital hypothyroidism screening in newborns starting from mid-1970s, there have been extensive human studies investigating the correlation between maternal exposure to EDCs and the newborn's thyroid function. Pituitary produced TSH levels are measured as a reflection of the function of the hypothalamic-pituitary-thyroid (HPT) axis. Any impact of EDCs on thyrotroph hormone production or feedback regulation would negatively affect this critical developmental axis. Multiple studies have shown alterations in newborn's TSH levels associated with exposure to different types of EDCs (Sun et al. 2022).

In addition to human studies looking at correlations between prenatal EDC exposure and TSH levels, dosing experiments using rodents have revealed more direct relationships between EDC exposure and thyrotroph functions. Prenatal exposure to hydraulic fracturing chemicals (UOG mixture at 300 μg/kg/day) in mice caused elevated TSH levels in adulthood (Kassotis et al. 2016). Perinatal exposure to glycophosphate based herbicide (5, 50 mg/kg/day) decreased TSH levels at P90 (de Souza et al. 2017). Di-n-hexyl phthalate (DnHP) and dicyclohexyl phthalate (DCHP) could also have long-term effects on both sexes in rats after prenatal exposure, but their effects are dose-dependent. DnHP (100 mg/kg/day) decreased TSH levels while DCHP increased TSH levels at a lower dosage (20, 100 mg/kg/day) and increased TSH levels at a higher dosage (500 mg/kg/day) (Goktekin and Barlas 2017). Similar periods of exposure to PCB-126 (20, 40 μg/kg) caused elevated fetal TSH levels at e20 (R.G. Ahmed et al. 2018). For longer exposure periods, prenatal and lactational exposure to DEHP (30, 300, 750 mg/kg/day) and propylthiouracil (PTU, 3 ppm) cause elevated TSH levels in prepubertal offspring (Bansal and Zoeller 2019; Dong et al. 2019). The effects of BPA exposure on thyrotrophs are more complicated depending on different exposure window, sex and timepoint of inspection. Prenatal exposure to BPA caused males to have decreased TSH at P15 (300 mg/kg/day) and females to have increased TSH at P90 (100, 300 mg/kg/day) (Delclos et al. 2014), however, other studies showed increased TSH in young offspring caused by prenatal and/or lactational BPA exposure (R. Ahmed et al. 2018; Mahmoudi et al. 2018).

Overall, although there has been extensive research looking at TSH levels in relation to developmental EDC exposure. In most cases, a mechanism of action for altered TSH levels was not studied. Hypotheses include interference with thyroid function and thyroid hormone production. This could lead to feedback-level alterations of TRH from the hypothalamus and TSH from the pituitary, along with thyroid hormone receptor signaling interference. Studies have shown that thyrotroph development and proliferation can be regulated by thyroid hormone, TRH, and estrogens (Malaguti et al. 2004; Tonyushkina et al. 2017), which can all be impacted by developmental EDC exposure. Future studies could focus on understanding the impacts of EDCs on thyrotrophs by discovering the mechanisms by which hormone signals in this pathway are impacted by EDC exposure. In addition, uncovering the direct developmental impact of EDC exposure on components of the HPT axis at different exposure levels may yield more consistent results and allow mechanisms to be inferred more readily.

Effects of developmental EDC exposure on somatotrophs

Interestingly, compared to the complicated effects of different EDCs on other pituitary cell types, current studies revealing the consequences of developmental exposure to EDCs on somatotrophs are somewhat consistent. Most developmental EDC exposures caused a decrease in GH levels in animals regardless of different exposure window (prenatal or postnatal), various timepoints of inspection (embryonic, pre-pubertal, and adult) and sex differences. Exposure to both PCB-126 (20, 40 μg/kg) and dexamethasone (DEXA, 0.2 mg/kg) starting from e1 decreased rat fetal GH levels at e20 (Ahmed 2016; R.G. Ahmed et al. 2018). As for a slightly different exposure window, prenatal and lactational exposure to sodium valproate (50 mg/kg) also decreased GH levels in prepubertal rats at P14 and P21 (A-G et al. 2021). One-dose exposure to TCDD (0.05 – 30 μg/kg) and 2,3,4,7,8-pentachlorodibenzofuran (PnCDF, 1-1000 μg/kg) at e15 also decreased GH levels, as well as *Gh* mRNA levels, in both fetal (e20) and peri-adolescent (PNW5) rats (Taura et al. 2014). As for long-term effects, 10-day exposure to furan (10, 20 mg/kg/day) from P1 to P10 decreased GH levels in adult male rats (Rehman et al. 2019).

Although most EDCs caused a decrease in GH levels, some other EDCs could have the opposite or more complicated effects in terms of somatotroph functions. Prenatal exposure to a hydraulic fracturing chemical (UOG mixture at 300 µg/kg/day) caused female rats to have increased GH levels during adulthood (Kassotis et al. 2016). Furthermore, GH levels of different sexes seem to respond to BPA exposure differently: early-life postnatal exposure in male rats (20, 40 µg/kg) cause a decrease in GH levels (R. Ahmed et al. 2018), while an increase was seen in females (2.5-6.25, 25-62 mg/kg) (Ramirez et al. 2012). However, the opposite effect observed from these two studies could also be due to a different exposure window- P1-P10 vs. P15-P30, different dosages, as well as different timepoints of inspection- P30 (immediately after the exposure) vs. 5 months (long-term effects). Other than changes in GH levels, prenatal and lactational exposure DEHP (10, 100 nM) was found to alter some properties of somatotrophs: the proportion of ERα- and ERβ- positive somatotrophs was decreased in adult female rats (Pérez et al. 2020).

In sum, many impact of developmental EDC exposure on GH levels is relatively consistent - most studies reported decreased GH levels regardless of the types of EDCs and experimental conditions. Since GH is crucial for multiple physiological systems in the body, especially during development, understanding the role of developmental EDC exposure in association with conditions related to GH deficiency is in dire need. Take growth hormone deficiency (GHD) or combined pituitary hormone deficiency (CPHD) as examples, most studies focused on genetic or trauma-induced causes of these conditions while very few of them looked at their relationships to EDC exposure, specifically during development when somatotrophs are not fully established and possibly sensitive to disruptions. Furthermore, the proliferation and differentiation of somatotrophs can be mediated by hormones including GHRH, thyroid hormone, glucocorticoids, and retinoic acid (Ellsworth and Stallings 2018), all of which could potentially be disturbed by developmental EDC exposure. One direction for future studies could be examining impacts of developmental EDC exposure on signaling pathways regulated by those hormones at somatotrophs.

Effects of developmental EDC exposure on corticotrophs

The effects of developmental EDC exposure on corticotrophs have been evaluated by quantifying serum ACTH levels or measuring pituitary *Pomc* mRNA levels. However, the relationship between developmental EDC exposure and corticotroph functions is less studied compared to other cell types. Prenatal and lactational exposure to sodium valproate (50 mg/kg) caused an increase in ACTH levels in prepubertal rats at the ages of P14 and P21 (A-G et al. 2021). Exposure to nonylphenol (2 μg/mL in drinking water) during a similar exposure window caused a long-term increase in ACTH levels in adult male rats (Chang et al. 2012). Besides, a shorter period of exposure (e10 to P7) to BPA (2 μg/kg) also caused increases in ACTH levels in adult male rats as well (Chen et al. 2014). Interestingly, a 7-day postnatal exposure (P0 to P7) to BPA (0.5 μg/kg/day) caused a decrease in pituitary *Pomc* mRNA level in mice at P7 (Eckstrum et al. 2018), which is contradictory to previous findings describing the change of ACTH level in adults. However, since ACTH is the product undergoing post-translational modification from POMC, the possibility of EDC exposure interfering with intermediate processes that caused different changes in ACTH and *Pomc* still needs further investigation.

Given the limited number of studies investigating the impacts of developmental EDC exposure on corticotrophs, little is known about the mechanisms of how EDCs impact corticotroph development. However, it’s an important area for research since studies have shown the association between developmental EDC exposure and changes in stress response (Kitraki et al. 2016; Gore et al. 2022), which could be related to organizational hypothalamic-pituitary-adrenal axis effects. Changes in TFs expression, specifically *Pitx1* and *Tbx19* that regulate the differentiation of corticotrophs, alterations in corticotrophs’ response to CRH and cortisol, and regulation of CRH receptor and glucocorticoid receptor expression in response to developmental EDC exposure could be directions for future study.

Effects of developmental EDC exposure on stem cells/progenitor cells

Unlike hormone-producing cells in the anterior pituitary, the impacts of developmental exposure to EDCs on pituitary stem/progenitor cells are quite understudied. So far, only one paper focused on revealing how developmental exposure to BPA (0.5, 50 μg/kg/day) could affect pituitary stem/progenitor cell properties. Prenatal exposure to BPA from e10.5 to e18.5 increased proliferation in what appeared to be the stem cell population surrounding the cleft while increasing gonadotroph number only in female offspring. This finding indicates that developmental exposure to BPA in mice could influence reproductive functions by affecting pituitary stem cell proliferation and differentiation into gonadotrophs, which could be followed by altered gonadotropin levels (Brannick et al. 2012).

Stem cells are the earliest type of cells appearing in the pituitary gland. It's important to understand the impacts of developmental EDC exposure on the pituitary stem/progenitor cells as they are responsible for forming the gland and regulating the population size of different cell types. To understand the direct impacts of EDCs on pituitary stem/progenitor cells, future studies could investigate different aspects including the expression of key TFs related to stem cells maintenance and proliferation like *Sox2*, *Sox9*, and Notch signaling. Additionally, the impacts on lineage driving TFs like *Tbx19*, *Pou1f1* and *Nr5a1* should also be explored.

Transgenerational effects of developmental EDC exposure

In recent years, increasing amount of studies focuse on understanding the transgenerational effects of EDC exposure (Rissman and Adli 2014; van Cauwenbergh et al. 2020; Martini et al. 2020), which are usually carried out by epigenetic inheritance from exposed individuals (F0) to subsequent generations, and the mechanisms include DNA modification, histone modification and non-coding RNAs (ncRNAs) (Rissman and Adli 2014). Transgenerational effects on reproductive functions is a hot research topic and multiple studies examined both male and female reproductive function alterations in the offspring (Brehm and Flaws 2019). However, very few studies looked specifically at transgenerational effects on pituitary functions, and we could only find limited results showing changes in gonadotropin and *Gnrhr* levels. Embryonic exposure to DEHP (500 mg/kg/day) from e11 until birth in the F1 generation caused F3 females to have significant increase in FSH and borderline increase in LH levels at 1 year of age (Brehm et al. 2018). Two-dose embryonic exposure to a PCB mixture (Aroclor 1221, 0.1, 1, 10 mg/kg) at e16 and e18 in the F1 generation caused a decrease in LH levels in F2 females during proestrus at age of P42 (Steinberg et al. 2008). F1 embryonic exposure to TCDD (500 ng/kg/day) from e8 to e14 caused an increase in LH levels in F3 females during estrus at age of P70 (Yu et al. 2019). Except for rodents, transgenerational effects of EDC exposure were also observed in other animal models. In medaka (Oryzias latipes), a 7-day embryonic exposure to BPA (100 µg/L in water) in F0 fish caused adult males from F2 generation to have increased *Gnrhr2* mRNA level, and exposure to 17α-ethinylestradiol (EE) caused them to have an increase in *Lhb* mRNA level (Thayil et al. 2020).

Most transgenerational EDC studies have focused on gonads and very few of them have investigated changes in the pituitary gland. Thus, more explorations on EDCs' transgenerational effects on the pituitary are in need, especially with regard to important mechanistic insight such as epigenetic modifications including DNA methylation and histone modifications. Specifically, epigenetic modifications in the pituitary have been extensively studied for their association with various types of pituitary adenomas, and the epigenetically modified genes include multiple tumor suppressor genes and imprinted genes (Pease et al. 2013; Hauser et al. 2019). However, there hasn't been any research focusing on possible changes in epigenetic modifications in the pituitary in response to developmental EDC exposure, which could be a new perspective for understanding mechanisms of EDCs' transgenerational effects on the pituitary gland.

Thesis aims

As the master gland regulating the body's endocrine system that is associated with multiple physiological functions, understanding the development of the pituitary gland, including the mechanisms that establish the proper functioning of different cell types in the gland, is critical as disruptions during development could result in abnormal pituitary functions and eventually lead to hypopituitarism. In this study, we investigated both an intrinsic and an extrinsic signal that impact pituitary development and proposed novel mechanisms of their actions.

In Chapter 2, we investigated the mechanisms by which NOTCH signaling regulates pituitary development and its downstream targets. We identified a novel marker for pituitary stem cells-glycoprotein hormone subunit alpha 2 (GPHA2). We hypothesized that GPHA2 is regulated by NOTCH signaling in the developing pituitary. We discussed its potential functions for keeping the undifferentiated state of stem cells as well as acting as a stem cell secreted paracrine signal through TSHR signaling pathway and possibly regulating the neighboring cells for postnatal pituitary maintenance.

In Chapter 3, we investigated the systemic and direct actions of the endocrine disrupting chemical DEHP (and its primary metabolite MEHP) on the developing pituitary. We hypothesized that DEHP/MEHP could interfere with androgen signals through the hypothalamic-pituitary-gonadal axis, and activate AhR signaling pathway directly on the pituitary to regulate the functions of gonadotrophs.

Chapter 4 represents an initial characterization of the pituitary impact of developmental exposure to an environmentally relevant phthalate mixture.

Our overall hypothesis for this project is that development of the pituitary gland can be regulated and affected by both intrinsic and extrinsic signals, and together they are essential for the proper development and functioning of the pituitary gland.

Figure and table

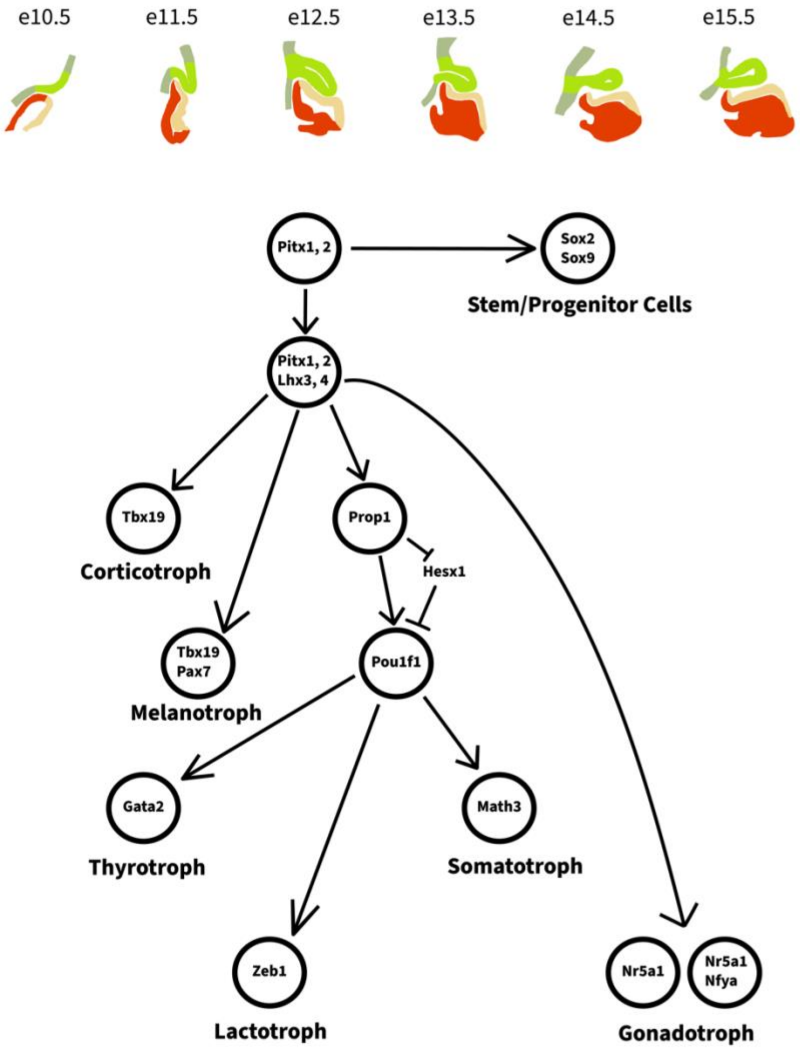

Figure 1.1 Development of the pituitary gland. A: the schematic diagrams of the developing pituitary in sagittal section from e10.5 to e15.5. Dark green: the diencephalon; light green: the infundibulum; yellow: the intermediate lobe; red: the progenitor cells of the presumptive anterior pituitary. B: major transcription factors regulating the proliferation and differentiation of different cell types in anterior and intermediate pituitary gland.

Table 1.1: Summary of EDCs effects on different cell types in developing pituitary

Gonadotrophs

Paper	**Animal**	**Sex**	**EDC**	**Dosing period**	**Detection time**	**Pituitary effect**
(Grandjean et al. 2012)	Human	Male	PCB		14 years	Decreased LH
(Kraugerud et al. 2012)	Lamb	Female	PCB	Prenatal and lactation	P60	Increased in GnRH-induced LH and FSH
(Kassotis et al. 2016)	Mouse	Female	Hydraulic fracturing chemical	e11 - birth	P85	Decreased LH FSH
(Ye et al. 2017)	Mouse	Male	Pyrethroid insecticide Cypermethrin	P7 - P21	P22	Increased LH FSH *Lhb Fshb*
(Pocar et al. 2012)	Mouse	Both	DEHP	In utero and lactation	P21	Increased *Lhb* in female, increased *Lhb* and *Fshb* in male
(Brannick et al. 2012)	Mouse	Female	BPA	e10.5 - e18.5	P1	Increased pit proliferation, increased gonadotroph cell number, increased *Lhb, Fshb, Gnrhr*
(Bellingham et al. 2009)	Ovine	Both	Sewage Sludge	Embryonic	e110	Decreased Kiss1, decreased kiss1-positive LHβ and ERα expressing gonadotrophs
(Li et al. 2020)	Quail	Female	DEHP	15 - 60 day before sex maturity	P60	Increased LH, decreased FSH
(Masutomi et al. 2004)	Rat	Both	Methoxychlor, MXC	e15 - P10	3, 11 week	Decreased LH FSH cells in males, only LH cells in females at wk3, increased FSH cells in females at wk11
(Lee et al. 2004)	Rat	Both	DBP	e15 - P21	P21, pnw11	Decreased FSH positive cells, increased LH positive cells in males and females
(Chen et al. 2017)	Rat	Male	DBP	e12 – e21	P4, P7, P14, P21, P28, P56	Decreased *Lhb* at P4, P7, P14, P21 Increased *Lhb* at P28, P56
(Yamamoto et al. 2005)	Rat	Male	PCB	e7 - e21	6, 15 weeks	Decreased LH and FSH

Table 1.1: Summary of EDCs effects on different cell types in developing pituitary (continued)

(Shobana et al. 2020)	Rat	Male	Hexavalent chromium	e15 - e21	P30	Increased LH, FSH
(Takeda et al. 2012)	Rat	Both	TCDD	e15 one dose	e18 – P0	Decreased LH, FSH and *Lhb, Fshb*
(Gámez et al. 2015)	Rat	Female	BPA	e0 - P21	P30	Increased LH
(Kariyazono et al. 2015)	Rat		TBT	e15 one dose	e20	Increased *Lhb*
(Abdel-Maksoud et al. 2015)	Rat	males	BPA + DEHP	e12 - e21	P35, P90	Increased LHβ FSHβ protein at P35, decreased at P90
(Hou et al. 2016)	Zebrafish	Female	Micro cyst in-LR	5-90 day post fertilization	P90	Increased *Lhb Fshb*

Lactotrophs

Paper	**Animal**	**Sex**	**EDC**	**Dosing period**	**Detection time**	**Pituitary effect**
(Kassotis et al. 2016)	Mouse	Female	Hydraulic fracturing chemical	e11 - birth	P85	Decrease PRL
(Kendig et al. 2012)	Mouse	Male	BPA	e0.5 - P36	P36	Increase PRL
(Li et al. 2020)	Quail	Female	DEHP	15-60 day before sex maturity	P60	Increase PRL
(Masutomi et al. 2004)	Rat	Both	Methoxychlor, MXC	e15 - P10	3, 11 week	Decrease PRL cells in males at wk3, increase PRL cells in females at wk11
(Shobana et al. 2020)	Rat	Male	Hexavalent chromium	e15 - e21	P30	Decreased PRL
(Johansson et al. 2016)	Rat	Female	AAmix	e13 - e19, P14 - P21	P22	Decreased PRL
(Lee et al. 2004)	Rat	Both	DBP	e15 - p21	P21, 11 week	Decreased PRL positive cells males and females at P21
(Pérez et al. 2020)	Rat	Female	DEHP	e0.5 - P21	P21, P75	Increase lactotroph, decreased ERα, β positive lactotroph

Table 1.1: Summary of EDCs effects on different cell types in developing pituitary (continued)

(Delclos et al. 2014)	Rat	Female	BPA	e6 - P90		Increase PRL
(Gomez et al. 2019)	Rat	Male	Glyphosat-based herbicide	e9.5 - P21	P21, P60	Increased PRL at P60
(Ramos et al. 2003)	Rat	Male	BPA	e8 - e23	P15, P30, P120	Increase PRL at P30

Thyrotrophs

Paper	**Animal**	**Sex**	**EDC**	**Dosing period**	**Detection time**	**Pituitary effect**
(Kassotis et al. 2016)	Mouse	Female	Hydraulic fracturing chemical	e11 - birth	P85	Increased TSH
(Goktekin and Barlas 2017)	Rat	Both	DHP, DCHP	e6 - e19	Adult	Decrease TSH by DHP in both sexes, Increase TSH by DCHP in at lower concentration, decrease TSH at higher concentration
(Dong et al. 2019)	Rat	Both	DEHP	e0 - P21	P7 P14 P21	Increased TSH
(Mahmoudi et al. 2018)	Rat	Both	BPA	e0 - P21	Young	Increased TSH
(R. Ahmed et al. 2018)	Rat		BPA	P15 - P30	P30	Increased TSH
(R.G. Ahmed et al. 2018)	Rat		PCB 126	e1 - e20	e20	Increased TSH
(Bansal and Zoeller 2019)	Rat	Both	PTU	e6 - P15	P15	Increased TSH, *Tshb*
(Delclos et al. 2014)	Rat	Both	BPA	e6 - birth	P15 P90	Male decreased TSH at P15, female increased TSH at P90
(de Souza et al. 2017)	Rat	Male	Glycophospbate based herbicide	e18 - P5	P90	Decreased TSH no change *Tshb*

Somatotrophs

Paper	**Animal**	**Sex**	**EDC**	**Dosing period**	**Detection time**	**Pituitary effect**
(Pérez et al. 2020)	Rat	Female	DEHP	e0.5 - P21	P21, P75	Decreased ERα, β positive somatotroph

Table 1.1: Summary of EDCs effects on different cell types in developing pituitary (continued)

(A-G et al. 2021)	Rat	Both	Sodium valproate	e8 - P21	P14, P21	Decreased GH
(Rehman et al. 2019)	Rat	Male	Furan	P1 - P10	Adult	Decreased GH
(R.G. Ahmed et al. 2018)	Rat		PCB 126	e1 - e20	e20	Decreased GH
(R. Ahmed et al. 2018)	Rat		BPA	P15 - P30	P30	Decreased GH
(Kassotis et al. 2016)	Mouse	Female	Hydraulic fracturing chemical	e11 - birth	P85	Increased GH
(Ahmed 2016)	Rat		DEXA	e1 - e20	e15, e20	Decreased GH
(Taura et al. 2014)	Rat	Both	PnCDF, TCDD	e15 one dose	e20, 5 week	Decreased GH, *Gh* mRNA
(Ramirez et al. 2012)	Rat	Femlae	BPA	P1 - P10	5 month	Increased GH

Corticotrophs

Paper	**Animal**	**Sex**	**EDC**	**Dosing period**	**Detection time**	**Pituitary effect**
(A-G et al. 2021)	Rat	Both	Sodium valproate	e8 - P21	P14, P21	Increased ACTH
(Eckstrum et al. 2018)	Mouse	Both	BPA	P0 - P7	P7	Decreased pituitary *Pomc*
(Chen et al. 2014)	Rat	Both	BPA	e10 - P7	P80	Increased ACTH in males
(Chang et al. 2012)	Rat	Males	Nonylphenol	e0 - P21	13-14 week	Increased ACTH

Stem Cells

Paper	**Animal**	**Sex**	**EDC**	**Dosing period**	**Detection time**	**Pituitary effect**
(Brannick et al. 2012)	Mosue	Female	BPA	e10.5 - e18.5	P1	Increase *Sox2* positive progenitor cells, decrease *Nr5a1*

Table 1.1: Summary of EDCs effects on different cell types in developing pituitary (continued)

Transgenerational Effects

Paper	Animal	Sex	EDC	Dosing period	Detection time	Pituitary effect
(Brehm et al. 2018)	Mouse	Female	DEHP	e11 - birth	F3 1 year	Increased FSH, borderline increased LH
(Steinberg et al. 2008)	Rat	Female	PCB mixture	F1 e16, e18	F2 P42	Decreased LH in F2 at proestrus
(Yu et al. 2019)	Rat	Female	TCDD	F1 e8 - e14	F3 P70	Increased LH in F3 at estrus
(Thayil et al. 2020)	Medka	Male	BPA, 17α-ethinylestradiol (EE)	F0 7-day embryonic exposure post fertiization	F2 adult fish	Increased *Gnrhr2* in F2 by BPA, increased *Lhb* in F2 by EE

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CHAPTER 2: GLYCOPROTEIN HORMONE SUBUNIT ALPHA 2 (GPHA2): A PITUITARY STEM CELL-EXPRESSED HORMONE ASSOCIATED WITH NOTCH2 SIGNALING

Abstract

As the major type of Notch receptor in the pituitary, *Notch2* is expressed in stem cells and is necessary for stem cell maintenance, proliferation and differentiation. However, the signaling pathways NOTCH2 engages to affect pituitary development remain unclear. In this study, we hypothesized that GPHA2 is downstream of NOTCH2 and contributes to stem cell actions. GPHA2 is a stem cell expressed gene in the cornea and is the alpha subunit of the hormone thyrostimulin, an agonist of the thyroid stimulating hormone receptor (TSHR). We mined single-cell RNA sequencing data of neonatal mouse pituitaries and found *Gpha2* mRNA is restricted to a subset of the stem cell population, that we confirmed by RNAscope *in situ* hybridization. Both qRT-PCR analysis and *in situ* hybridization showed a significant reduction of *Gpha2* mRNA in *Notch2*-cKO pituitaries compared with wildtype (WT) littermates, which indicates the expression of *Gpha2* in the pituitary is associated with *Notch2*. We then investigated the possible functions of GPHA2. Neonatal and adult pituitaries treated with a GPHA2 peptide showed increased pCREB expression. In dissociated adult primary pituitary cells, this induction was reversed by co-treatment with a TSHR inhibitor. These data suggest GPHA2 activates the TSHR signaling pathway and this downstream signaling could be a key component in NOTCH2's regulation of pituitary development.

Introduction

Hypopituitarism is a condition defined as a shortage of one or more kinds of pituitary hormones, which can then lead to various symptoms in the individual, including growth restriction, metabolic deficiencies, and reproductive dysfunction (Bosch i Ara et al. 2021). Genetic causes of hypopituitarism have been extensively investigated and multiple genes have been identified to be associated with this condition. Most of them are critical transcription factors regulating the development of the pituitary including *Lhx3*, *Lhx4*, *Prop1*, *Pit1/Pou1f1*, and *Hesx1* (Fang et al. 2016). However, other than the already known genetic mutations leading to hypopituitarism, the causes of over 80% of the cases remain unclear (Fang et al. 2016). This indicates other mechanisms yet to be discovered could be involved with this condition.

The NOTCH signaling pathway is crucial for the pituitary development. Specifically, the NOTCH signaling pathway is necessary for mediating pituitary proliferation and differentiation, as NOTCH target gene *Hes1* mutant mice had reduced pituitary proliferation and cell specification during embryogenesis (Raetzman et al. 2007). Besides, the NOTCH signaling is also important for postnatal pituitary progenitor cell maintenance and expansion. Treatment with NOTCH inhibitor, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT), resulted in decreased progenitor cell number and postnatal proliferation, as well as decreased postnatal anterior pituitary volume (Nantie et al. 2014). Among different NOTCH receptors, *Notch2* is expressed in the pituitary gland and specifically in pituitary stem/progenitor cells (Raetzman et al. 2004). Our lab's previous study reported that the phenotypes of a *Notch2* conditional knockout mouse model (*Notch2*-cKO) are very similar to *Prop1* mutant mice, which is a known model for hypopituitarism (Bartke and Brown-Borg 2004). The shared phenotypes between *Notch2*-cKO mice and *Prop1* mutant mice include anterior pituitary hypoplasia and reduced lactoroph, thyrotroph and somatotroph differentiation, leading to reduced body size compared to littermates (Nantie et al. 2014). Although we see the hypopituitarism-like phenotypes in *Notch2*-cKO mice, the downstream targets of *Notch2* signaling in the pituitary and the mechanisms of how they regulate the functions of the pituitary that lead to the phenotype remain unclear.

To further investigate the downstream targets of NOTCH signaling pathway in the pituitary, our lab performed microarray analysis on *Notch2*-cKO mice and found mRNA of the gene encoding glycoprotein hormone subunit alpha 2 (*Gpha2*) was significantly decreased in *Notch2*-cKO pituitaries compared to WT pituitaries (unpublished data). *Gpha2* has been reported to be mostly expressed in the eye and pituitary gland in rodents (Nagasaki et al. 2006). As of its functions, GPHA2 was recently reported as a novel marker for corneal quiescent stem cells in mice (Altshuler et al. 2021), and it was found to support the undifferentiated state of corneal stem cells in human (Collin et al. 2021). GPHA2 also can heterodimerize with glycoprotein hormone subunit beta 5 (GPHB5). Together they act as hormone thyrostimulin and activate TSHR signaling in multiple cell lines and organs including the ovary (Nagasaki et al. 2006; Okada et al. 2006; Sun et al. 2010). Activation of TSHR by thyrostimulin involves multiple downstream signaling pathways including PKA/CREB, PI3K/AKT, MAPK/ERK, and trans-activated EGFR signaling (Huang et al. 2016). However, the target receptors of GPHA2 and the downstream signaling pathways that it activates as a monomer in the pituitary gland are unknown.

In this study, we hypothesize: 1) *Gpha2* is one of the downstream target gene of NOTCH signaling in the pituitary; and 2) GPHA2 acts through TSHR in the pituitary in a paracrine manner. We aim to: 1) understand the expression pattern and regulation of GPHA2 in the developing pituitary in mice, possibly through the NOTCH signaling pathway; and 2) discover the target receptor and signaling pathway of GPHA2 in the pituitary and its possible role in the pituitary development. We mined single cell RNA sequencing (scRNA-seq) datasets from mouse developing and adult pituitaries, detected the expression of *Gpha2* mRNA in *Notch2*-cKO mouse pituitaries compared to WT pituitaries using both qRT-PCR and *in situ* hybridization (ISH), and treated pituitaries with GPHA2 peptide and/or TSHR inhibitors to investigate downstream signaling pathways that GPHA2 activates in the pituitary.

Materials and Methods

Mice

CD-1 mice. CD-1 mice were originally obtained from Charles River and were bred in house at the animal facility of the University of Illinois Urbana-Champaign for all experiments described. Sex was confirmed by visual inspection and SRY genotyping of the tail using the forward primer: 5'- TGCAGCTCTACTCCAGTCTTG-3' and reverse primer: 5'- GATCTTCATTTTTAGTGTTC-3'. The University of Illinois Urbana-Champaign Institutional Animal Care and Use Committee approved all procedures.

***Notch2*-cKO mice.** $Notch2^{fl/fl}$ mice were bred to $Foxg1^{+/cre}$ mice, both purchased from the Jackson Laboratory, to generate $Notch2^{+/fl}$,$Foxg1^{+/cre}$ and $Notch2^{+/fl}$,$Foxg^{+/+}$ mice. Those two genotypes were then bred to generate *Notch2*-cKO mice. Detailed information was described in a previous publication (Nantie et al. 2014).

Single cell RNA sequencing analysis

Mouse single cell RNA Seq (scRNA-seq) data were downloaded from Gene Expression Omnibus (GEO) website under accession number GSe142074, GSe120410 and GSe151958. scRNA-seq data downloaded from GEO were analyzed in R using Seurat 4.0.1 package. For cell filtering, we filtered out cells with a gene number fewer than 1000 or more than 5000, and only kept cells with less than 10% mitochrondrial genes. Top 2000 variable genes were identified using "FindVariableFeatures" function with "vst" method. After normalizing the data, we performed principal component analysis (PCA) and the top 10 PCs were selected for 2-dimension reduction by uniform manifold approximation and projection (UMAP). We identified the clusters using known marker genes described in the original paper that generated and analyzed the dataset (L.Y.M. Cheung et al. 2018; Cheung and Camper 2020; Ruf-Zamojski et al. 2021).

In situ hybridization (ISH)

A probe for *Gpha2* was generated by PCR amplification from mouse genomic DNA using forward primer: 5’-ATGCCCATGGCACCACGAGTC-3’, and reverse primer: 5’-AGGAGACTGCTGAGAGGGCC-3’. The sequence was then cloned into pGEM®-T Easy Vector using TA cloning kit (Promega) following the manufacturer’s instructions and antisense sequence was amplified by PCR reaction using forward primer and T7 primer and purified using Wizard® SV Gel and PCR Clean-Up System (Promega) following manufacturer’s instructions. Probes were then labeled with DNA digoxigenin-UTP (DIG) and purified using ammonium acetate (NH4Ac) and diluted in hybridization solution.

Heads of mice at postnatal day 4 (P4) of were fixed in 3.7% formaldehyde (Sigma Aldrich) diluted in PBS and embedded in paraffin for sectioning. After sectioning, slides were deparaffinized, rehydrated, and incubated in PBS with 0.3% Triton X-100 (Sigma-Aldrich) then permeabilized using proteinase K (Life Technologies). Samples were then postfixed using 3.7% formaldehyde (Sigma-Aldrich) and acetylated for 10 minutes in triethanolamine (Sigma-Aldrich) buffer containing 0.25% acetic anhydride (Sigma-Aldrich). After acetylation, sections were incubated in hybridization solution at 56°C for 2 hours. After incubation, DIG-labeled probes for *Gpha2* were denatured at 95°C for 2 minutes and added to the slides in hybridization solution at hybridization temperature overnight, with no-probe hybridization solution as a control. The next day, samples were treated with a solution of 50% formamide (Sigma-Aldrich) for 15 to 45 minutes at the hybridization temperature, followed by blocking and then application of anti-DIG Fab fragments (Sigma-Aldrich) for 2 hours at room temperature. Slides were then washed, treated with chromagen buffer solutions (Sigma-Aldrich) of increasing pH, and developed at room temperature overnight in chromagen buffer containing nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Quantification of ISH staining was adapted from a previously published paper using Image J (Dobrzycki et al. 2018). To compare the signal intensity between *Notch2*-cKO and WT pituitaries, 4 sections from different individuals of each group were imaged using a Leica DM2560 LED optical microscope at 40x magnification and used for analysis.

RNAscope in situ hybridization

Whole embryos at embryonic day 11.5 and 13.5 or whole heads of mice at P4 were fixed in 3.7% formaldehyde (Sigma Aldrich) diluted in PBS and embedded in paraffin for sectioning. After sectioning, RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics, ACD) was used following the manufacturer's instructions for formalin fixed paraffin embedded tissues. Probes for *Sox2* and *Gpha2* were purchased from ACD.

In vitro pituitary culture

Pituitaries were removed from CD-1 mice at the age of P1. Pituitaries (n = 2-3) from the same sex were cultured on 96-well plate (Millipore) in DMEM/F12 medium containing 10% charcoal stripped Fetal B ovine Serum (FBS, Sigma), and 10,000 IU Penicillin/10,000 µg/ml Streptomycin (P/S, Fisher Scientific). Pituitaries were allowed to recover for 1 hour and then treated with GPHA2 peptide (Sino Biological) at 1 µg/mL or forskolin (Sigma Aldrich) at 0.82 µg/mL for 30 minutes. Pituitaries were then collected for cryosectioning and immunohistochemistry.

Immunohistochemistry (IHC)

Pituitaries from *in vitro* culture were fixed in 3.7% formaldehyde (Sigma Aldrich), diluted in PBS for 20 minutes, and cryoprotected in 30% sucrose solution in PBS overnight. Pituitaries were then snap frozen in O.C.T compound (TissueTek, CA, USA). Serial coronal sections (10 µm) were collected and mounted on slides while they were kept frozen. Frozen sections were then thawed and fixed in 3.7% formaldehyde (Sigma Aldrich), followed by a PBS wash and, a 5-minute hot 1X sodium citrate (10 µM, pH=6.0) bath at boiling temperature. Slides were then blocked in blocking solution (5% normal donkey serum, 3% bovine serum albumen and 0.5% triton-X100 diluted in sterile PBS) for 1 hour and incubated in primary antibody phospho-CREB (pCREB) antibody (rabbit, 1:400; Upstate) at 4°C overnight. Slides were then washed with PBS with 0.1% tween (Fisher Scientific) and incubated with Cy3-conjugated secondary antibody (1:250, Jackson ImmunoResearch). Slides were then mounted with mounting media containing 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; Sigma-Aldrich) and visualized using a Leica DM2560 LED optical microscope. Images were taken using a Retiga 2000R color camera and acquired using Q-Capture software. Images were then processed with Adobe Photoshop 2022 and pCREB signal intensity was quantified using ImageJ software. For quantification, 2 pictures of each side of anterior lobe from the same pituitary were taken and three pituitaries in total were used for analysis.

Dissociated pituitary cell culture

Pituitaries were removed from adult CD-1 mice. Pituitaries (n = 6-7) from both males and females combined were treated with 0.25% trypsin for 30 minutes in 37°C followed by 15 minutes treatment in DNase. After inactivation of trypsin with equal volume of HBSS (Corning) + 10% donkey serum, pituitary tissues were triturated with wide and narrow bore Pasteur pipets. Cells were centrifuged for 5 minutes at 150 x g and resuspended in DMEM/F12 medium containing 10% charcoal stripped FBS, and 1% P/S. Cell numbers were counted using hemacytometer and plated in 6-well plate with density of 1 million cells/well. Cells were allowed to recover and attach to the plate overnight. The media were replaced by fresh DMEM/F12+ charcoal stripped FBS+P/S media containing TSHR inhibitor ML224 (MedChemExpress) at final concentration of 1 µg/mL or equal volume of DMSO as control. After 30 minutes pre-treatment of TSHR inhibitor/DMSO, GPHA2 peptide or PBS was added at the final concentration of 250 ng/mL and cells were incubated for another 30 minutes. Cells were then collected for western blot analysis.

Western blot

Cells were collected on ice and lysed using radioimmunoprecipitation assay buffer (RIPA). Protein fragments were obtained by centrifugation and total protein concentration was measured via a BCA protein assay kit (Thermo Fisher) according to the manufacturer's instructions. Protein samples (10 µg) were mixed with RIPA buffer and 3x Laemmli loading dye and then heated at 55°C for 10 minutes. Samples were loaded on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (BioRad). The membrane was blocked for two hours in 5% non-fat milk in tris buffered saline (TBS) then incubated with pCREB antibody (rabbit, 1:1000; Upstate), CREB antibody (rabbit, 1:500, Cell Signaling) or α-tubulin antibody (mouse; 1:5000; Sigma-Aldrich) as control in 1% milk with 0.2% tween for overnight at 4°C. Secondary goat anti-rabbit or anti-mouse antibody (1:5000) conjugated to IRdye 800cw (LI-COR Biosciences) was added at room temperature for 1 hour. Membranes were then imaged with the Odyssey infrared imaging system (LI-COR). The relative protein levels were analyzed by ImageJ. For quantification, pCREB or CREB protein levels were normalized to α-tubulin as housekeeping protein by dividing the intensity of pCREB/CREB band to α-tubulin band in the same lane. All experimental groups were then compared with the control group by dividing each group's normalized intensity to control normalized intensity and fold change values for each treatment group were obtained and used for statistical analysis. The detailed methods adapted for quantification were described in this paper (Pillai-Kastoori et al. 2020). Three individual sets of experiments were performed.

RNA isolation and cRNA preparation

Pituitaries at age of P1 from *Notch2*-KO mice and WT littermates were homogenized and RNA was isolated using the RNAqueous-Micro kit (Invitrogen) according to the manufacturer's protocol. Total RNA was reverse-transcribed according to the manufacturer's instructions using the ProtoScript Strand cDNA Synthesis kit (New England Biolabs) as previously described (Moran et al. 2012).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Oligonucleotide primers for *Gpha2* (Life Technologies) were used to amplify gene-specific transcripts by qRT-PCR. The expression levels of genes of interest were normalized to *Actb* mRNA levels. *Actb* is a stable housekeeping gene for qRT-PCR normalization that we confirmed had consistent expression levels across different genotypes (Nantie et al. 2014). The data were analyzed using the standard comparative cycle threshold value method ($2^{-\Delta\Delta Ct}$) as previously described (Goldberg et al. 2011). PCR primer sequences for *Gpha2* forward: 5'-AGGCTGCCACTTGCACCCC-3'; *Gpha2* reverse: 5'-CTCCCCACGCTGGTTCCC-3'; *Actb* forward: 5'-GACATGGAGAAGATCTGGCA-3'; *Actb* reverse: 5'-GGTCTCAAACATGATCTGGGT-3'.

Statistical analysis

All data were presented as mean +/- SEM. Statistical significance was determined using Student's t-test or one-way ANOVA followed by Dunnett's or Tukey's multiple comparisons test as noted in the figure legends. P values less than 0.05 were considered significant. All analyses were performed using Graph Pad Prism 9.2.0.

Results

Gpha2 mRNA was expressed in subset of stem cells in developing mouse pituitary

From the P4 mouse pituitary scRNA-seq dataset, we identified different clusters of cells using marker genes described in the original paper (Cheung and Camper 2020). We found 3 different stem cell clusters (Stem1, Stem2 and Stem3) using stem cell marker *Sox2* (Figure 2.1 A-B). Using proliferation marker *Mki67*, we identified proliferating stem cells- Stem3, and non-proliferating stem cells- Stem1 and Stem2 (Figure 2.1 C-D). *Notch2* was mostly expressed in Stem1 and Stem3 (Figure 2.1 E-F), and *Gpha2* was expressed in the same clusters as *Notch2*, but only in a subset of cells in those clusters and mostly in the non-proliferating Stem1 (Figure 2.1 G-H). *Gphb5* was not detected in the datasets.

From the adult mouse pituitary scRNA-seq dataset (Cheung et al. 2018), the stem cell population was restricted to 1 cluster (Figure 2.2 A-B) as the proliferating stem cells were not detectable anymore (Figure 2.2 C-D). *Notch2* (Figure 2.2 E-F) and *Gpha2* (Figure 2.2 G-H) were still present in adult pituitary stem cells. Again, *Gphb5* was not detected.

Following the scRNA-seq analysis, we examined the expression of *Gpha2* mRNA in developing pituitaries at embryonic day 11.5 (e11.5), e13.5 and P4, and observed co-localization of *Gpha2* and *Sox2* signals at all ages (Figure 2.3). Specifically, in P4 pituitaries, we observed a few *Sox2*+ cells expressing strong *Gpha2* signals only in the intermediate lobe (Figure 2.3 C). Other *Sox2+/Gpha2+* cells were scattered in the anterior lobe parenchyma, with relatively weaker *Gpha2* signals compared to *Gpha2*+ cells in the intermediate lobe (Figure 2.3 D). *Gpha2* was not expressed in the *Sox2*+ stem cells along the cleft (Figure 2.3 C-D).

Gpha2 expression was associated with Notch2 in the pituitary

Given the results from scRNA-seq analysis showing *Gpha2* and *Notch2* mRNAs were expressed in the same clusters of cells, we examined the expression of *Gpha2* mRNA in *Notch2*-cKO mouse pituitaries compared to WT pituitaries. Both qRT-PCR analysis (Figure 2.4 A) and ISH staining (Figure 2.4 B-C) followed by quantification (Figure 2.4 D) showed *Gpha2* expression levels were significantly decreased in *Notch2*-cKO pituitaries compared to WT pituitaries, indicating the expression of *Gpha2* in the pituitary could be regulated by the NOTCH signaling pathway.

TSHR was expressed in mouse pituitary and its signaling was activated by GPHA2

To identify the target receptor and downstream signaling pathway that GPHA2 activates in the pituitary, we examined the possible target of GPHA2 in the pituitary, TSHR. It is expressed in the pituitary (Nagasaki et al. 2006) and it has been reported to be the receptor for thyrostimulin (Nakabayashi et al. 2002; Sun et al. 2010; Huang et al. 2016). However, the cells expressing TSHR in the pituitary remained unclear. From adult mouse pituitary scRNA-seq dataset (Ruf-Zamojski et al. 2021), we observed expression of *Tshr* was relatively sparse across different cell types, with a concentration in the pars tuberalis (PT, rostral tip thyrotropes) (Figure 2.5).

As a first step in determining if GPHA2 could signal in the pituitary, we performed *in vitro* pituitary explant cultures with GPHA2 or forskolin as a positive control, followed by IHC with pCREB antibody (Figure 2.6 A). CREB phosphorylation is a known downstream target of the TSHR signaling pathway (Huang et al. 2016). Quantification of IHC staining showed a significant increase of pCREB signal in pituitaries treated with either GPHA2 or the known adenylyl cyclase activator forskolin (Figure 2.6 B), indicating induction of pCREB by GPHA2. To test the hypothesis that induction of pCREB was through TSHR signaling, we then performed dissociated pituitary cell cultures, treating the cells with GPHA2 and/or TSHR inhibitor and examined pCREB levels using western blots. Western blot analysis showed significant induction of pCREB in cells treated with GPHA2 peptide, and the induction was inhibited by co-treatment with TSHR inhibitor (Figure 2.7 A). As a control protein, CREB remained unchanged in all experimental groups (Figure 2.7 B).

Discussion

Overall, these data describe a novel stem cell gene, *Gpha2*, in the mouse pituitary that changes in parallel with NOTCH signaling. The scRNA-seq analysis allowed us to identify different types of stem cells in neonatal pituitaries. The heterogeneity of stem cells, including with regard to proliferation, indicates they could be responsible for different functions during pituitary development. Compared with adult pituitaries, proliferating stem cells are exclusive to neonatal pituitaries. The scRNA-seq analysis identified *Gpha2* expression in stem cells in the pituitary. We demonstrated by RNAscope ISH that *Gpha2* was expressed in a subset of stem cells in the intermediate and anterior lobes. The fact that *Notch2* and *Gpha2* are expressed in both neonatal and adult pituitaries indicates they may play essential roles in stem cell maintenance and/or differentiation. Previous studies have reported that *Gpha2* is a marker for quiescent corneal stem cells (Altshuler et al. 2021) and functionally, it supports the undifferentiated state of the stem cells (Collin et al. 2021). Taking the scRNA-seq data and previous findings together, future studies investigating the functions of *Gpha2* and *Notch2* in the pituitary could focus on their potential role in maintaining the quiescent and undifferentiated state of pituitary stem cells.

As for the relationship between *Notch2* and *Gpha2* in the pituitary, our current data suggest *Gpha2*'s expression in the pituitary is regulated by *Notch2* as they are expressed in the same clusters of stem cells and *Gpha2*'s expression was significantly decreased in *Notch2*-cKO pituitaries. The reduction of *Gpha2* mRNA levels in the *Notch2*-cKO pituitary could be due to direct transcriptional regulation of *Gpha2* by NOTCH2 signaling. Alternatively, *Gpha2* mRNA may be reduced due to the decrease in stem cell population caused by loss of *Notch2*, as we've previously reported the loss of stem cells in *Notch2*-cKO pituitaries after birth (Nantie et al. 2014). However, whether *Notch2* directly or indirectly regulates *Gpha2* remains unknown. To further understand if *Gpha2* is a direct target of NOTCH signaling, future studies could examine possible binding of NOTCH signaling effector RBPJ (Castel et al. 2013) within the *Gpha2* promoter region.

In terms of GPHA2's downstream targets in the pituitary, we demonstrated activation of the TSHR signaling pathway by GPHA2. Neonatal pituitaries and dissociated adult pituitary cells treated with GPHA2 peptide had induced pCREB levels. In dissociated pituitaries, the induction of pCREB by GPHA2 was inhibited by co-treatment with TSHR inhibitor. This is the first demonstration that, in the pituitary, monomeric GPHA2 could signal through TSHR. This finding provided us several perspectives for understanding GPHA2 functions in the pituitary. As a peptide secreted from stem cells, GPHA2 may function as a paracrine factor to regulate neighboring progenitor cells through the TSHR signaling pathway (Figure 2.8). This possible paracrine role of GPHA2 in pituitary is inspired by a recent study reporting that pituitary stem cells were able to secrete paracrine WNT signals and regulate the neighboring cell's proliferation during postnatal pituitary expansion (Russell et al. 2021). Similar to WNT signaling that is crucial for pituitary expansion during early development (Chambers et al. 2013; Osmundsen et al. 2017), NOTCH signaling is a parallel pathway that plays important roles in regulating pituitary development and maintenance. Although we do not know if GPHA2 is solely functioning in a paracrine manner, we have identified TSHR as one target through which GPHA2 exerts its functions and this receptor is not in stem cells. It is also possible that GPHA2 has a role directly in pituitary stem cell quiescence as it does in the cornea (Altshuler et al. 2021) through a yet to be identified pathway. Future studies are necessary to fully understand the mechanisms of GPHA2's actions in the pituitary.

Acknowledgements

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Figures

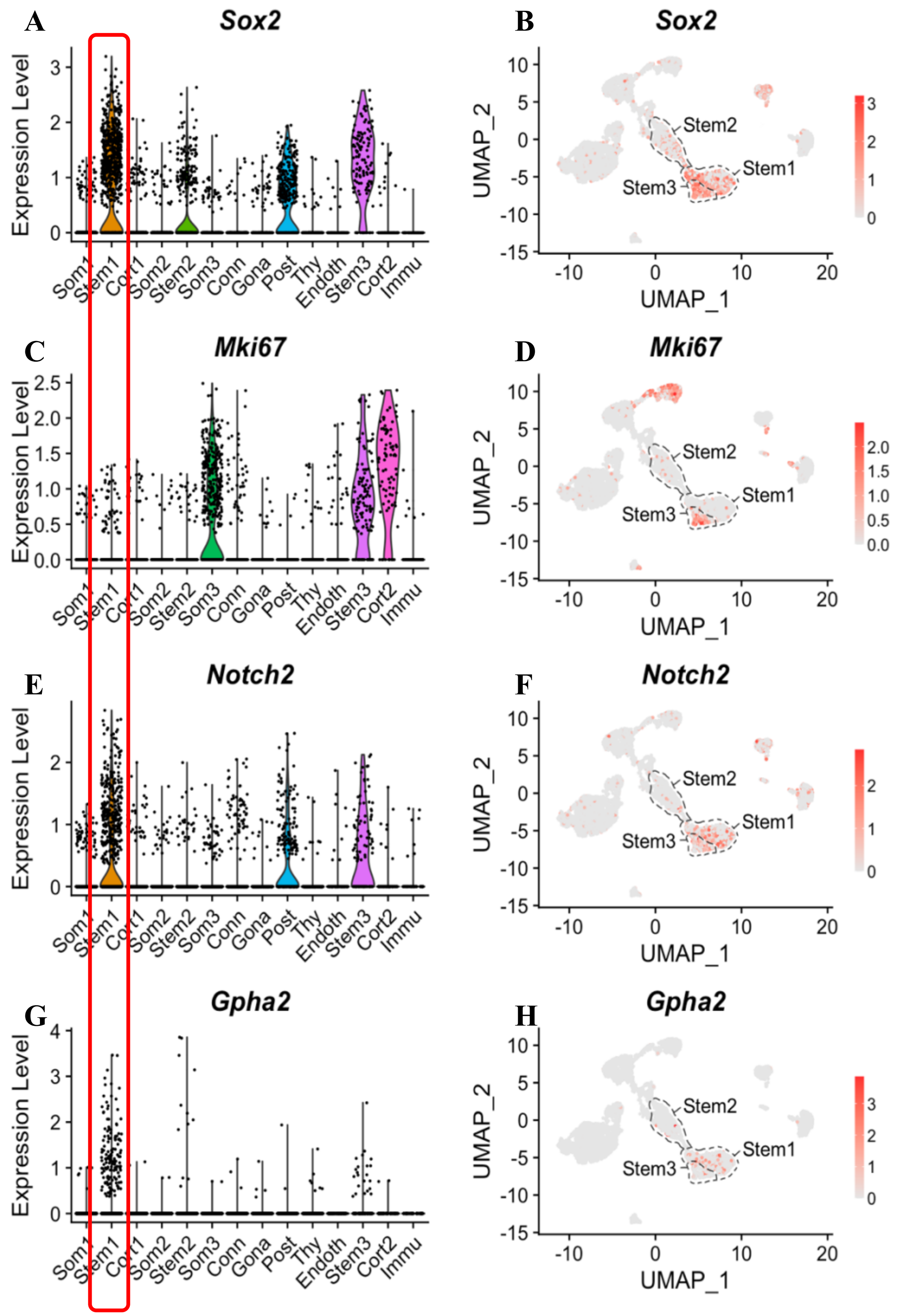

Figure 2.1 scRNA-Seq analysis showing *Sox2* (A-B), *Mki67* (C-D), *Notch2* (E-F) and *Gpha2* (G-H) expression levels across different cell types in P4 pituitary. Som: somatotrophs; Stem: stem cells; Cort: corticotrophs; Conn: connective tissue; Gona: gonadotrophs; Post: posterior pituitary cells; Thy: thyrotrophs; Endoth: endothelial cells; Immu: immune cells. Sequencing data were downloaded from GEO under accession code: GSE142074 and plots were generated using Seurat package in R.

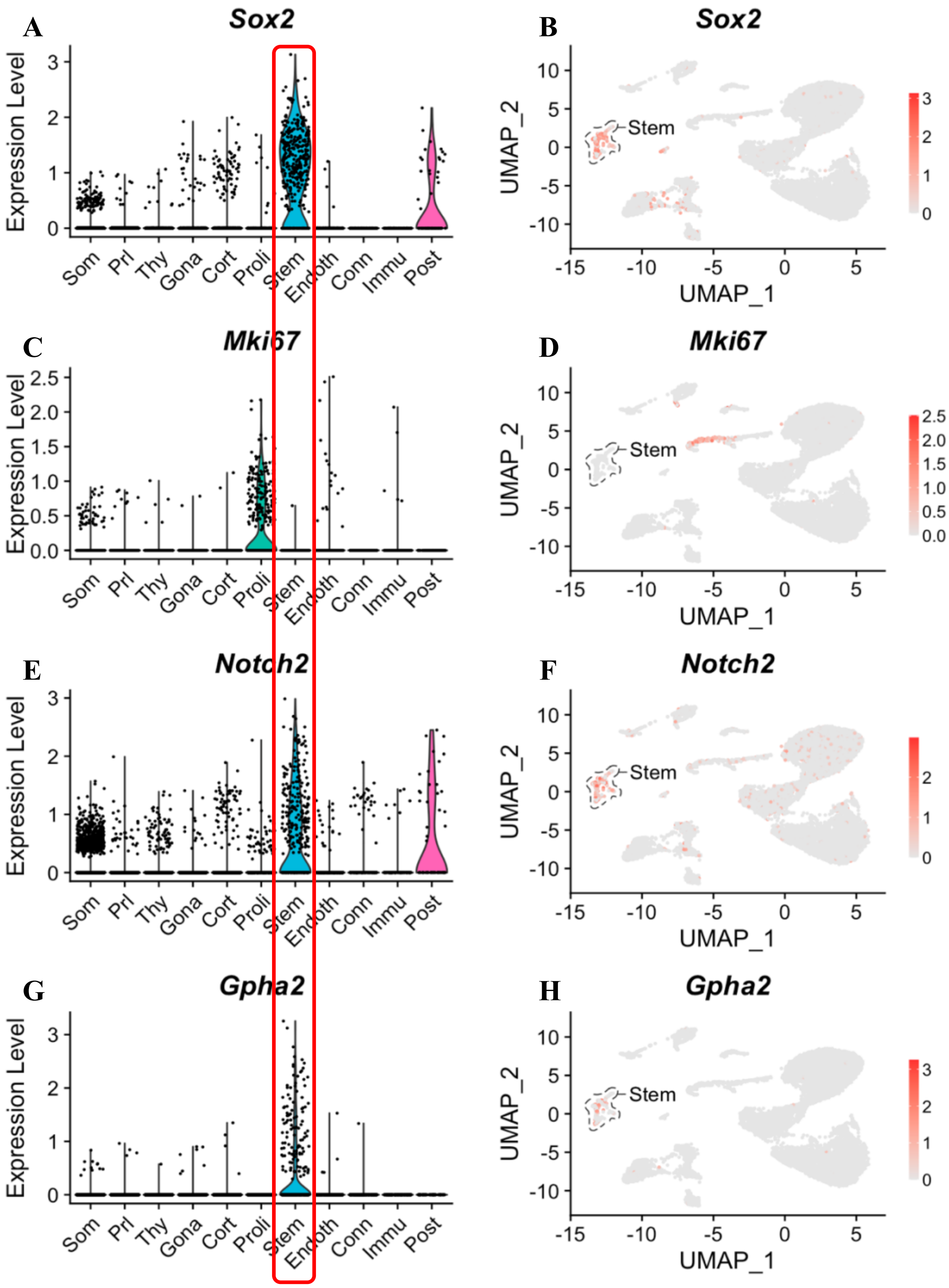

Figure 2.2 scRNA-Seq analysis showing *Sox2* (A-B), *Mki67* (C-D), *Notch2* (E-F) and *Gpha2* (G-H) expression level across different cell types in adult pituitary. Som: somatotrophs; Stem: stem cells; Cort: corticotrophs; Conn: connective tissue; Gona: gonadotrophs; Post: posterior pituitary cells; Thy: thyrotrophs; Endoth: endothelial cells; Immu: immune cells; Proli: proliferating cells. Sequencing data were downloaded from GEO under accession code: GSE120410 and plots were generated using Seurat package in R.

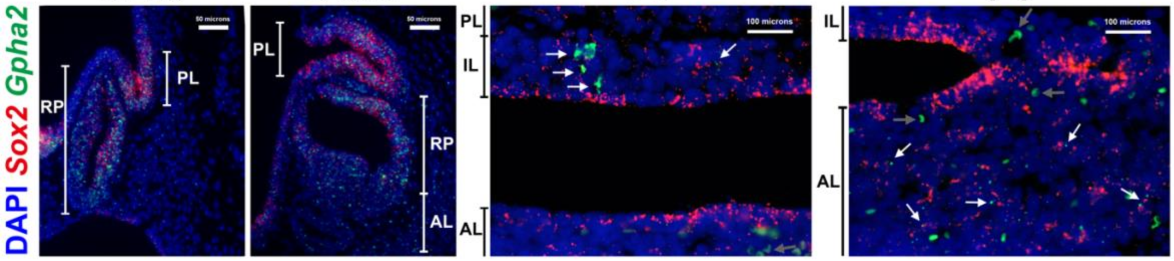

Figure 2.3 RNAscope ISH staining showing *Gpha2* (green) and *Sox2* (red) mRNA overlapped with DAPI (blue) staining the nuclei in the pituitary at e11.5 (A), e13.5 (B) and P4 (C, D). PL: posterior lobe; RP: Rathke's pouch; IL: intermediate lobe; AL: anterior lobe. White arrows indicate *Gpha2* signal, gray arrows indicate red blood cells.

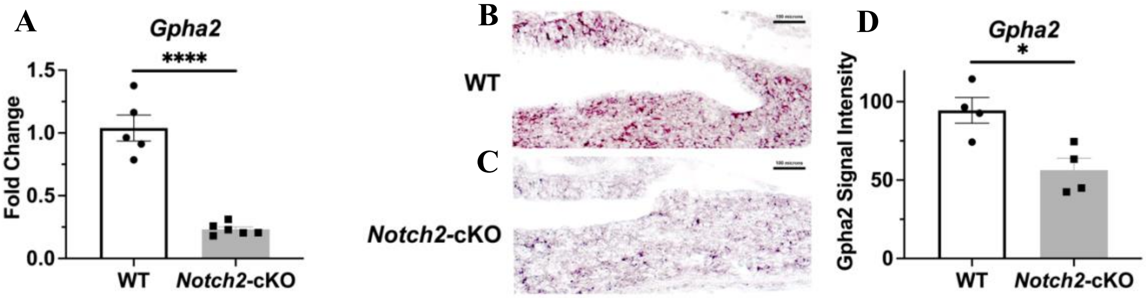

Figure 2.4 qRT-PCR (A) and ISH (B-D) showing decreased *Gpha2* mRNA level in *Notch2*-cKO mouse pituitaries compared to WT mouse pituitaries. Quantification of ISH was done using ImageJ software measuring the *Gpha2* signal intensity normalized to background (D). For A and D, the graph represents the mean +/− SEM (n = 4-6). Student's t-test was performed and *P < 0.05, ****P<0.0001

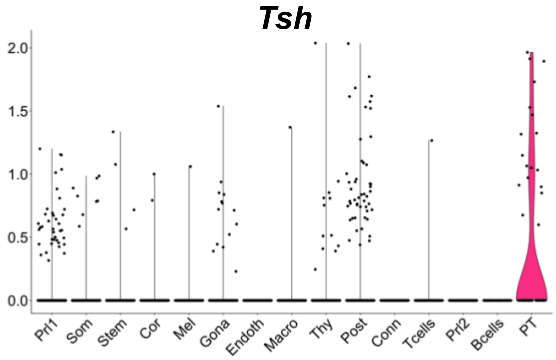

Figure 2.5 scRNA-Seq analysis showing *Tshr* expression level across different cell types in adult pituitary. Som: somatotrophs; Stem: stem cells; Cort: corticotrophs; Conn: connective tissue; Gona: gonadotrophs; Post: posterior pituitary cells; Thy: thyrotrophs; Endoth: endothelial cells; Mel: melanotrophs; Macro: macrophages, Tcells, Bcells: T cells, B cells; PT: pars tuberalis. Sequencing data were downloaded from GEO under accession code: GSE151958 and plots were generated using Seurat package in R.

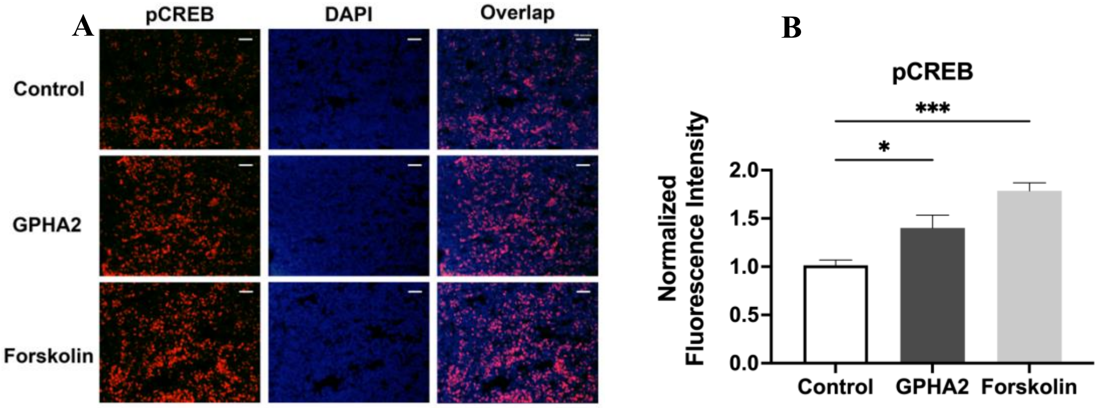

Figure 2.6 IHC staining showing pCREB overlapped with DAPI staining in the pituitaries at P1 (A). Quantification of IHC was done using ImageJ software measuring the pCREB signal intensity normalized to background (B) and the graph represents the mean +/− SEM (n = 6). One-way ANOVA followed by Dunnett's multiple comparisons test was done. Asterisk (*) represents significant difference from vehicle control. GPHA2: 1 μg/mL; Forskolin: 0.82 μg/mL. One-way ANOVA P = 0.0003, *P<0.05, ***P<0.0005 by Dunnett's multiple comparisons test.

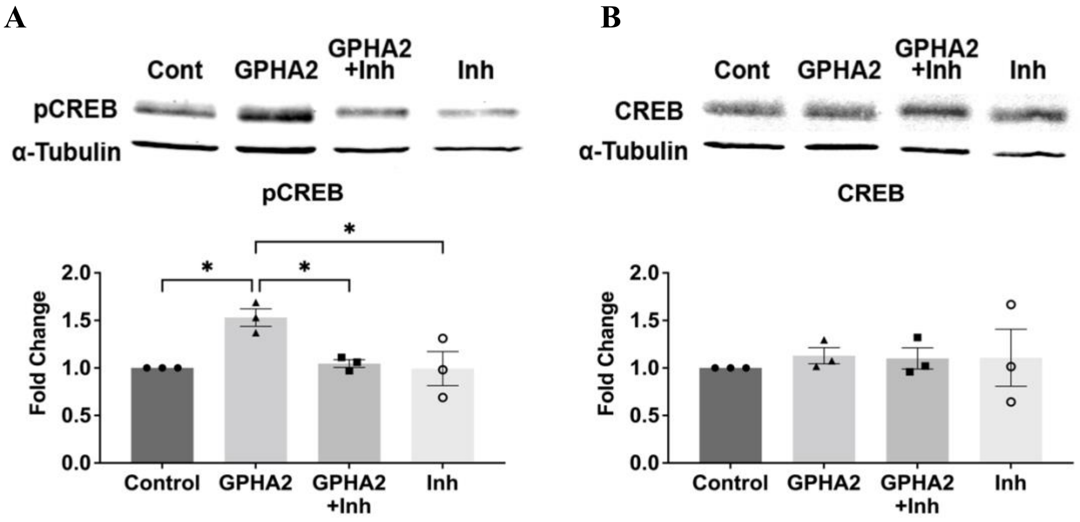

Figure 2.7 Western blot analysis showing expression levels pf pCREB (A) and CREB (B) proteins relative to α-tubulin in different treatment groups. GPHA2: 250 ng/mL; Inh: TSHR inhibitor, 1 μg/mL. For quantification, fold change levels were used for statistics analysis and the graph represents the mean +/− SEM (n = 3). One-way ANOVA followed by Tukey's multiple comparisons test was done. Asterisk (*) represents significant difference from vehicle control, One-way ANOVA P = 0.0163, *P<0.05 by Tukey's multiple comparisons test.

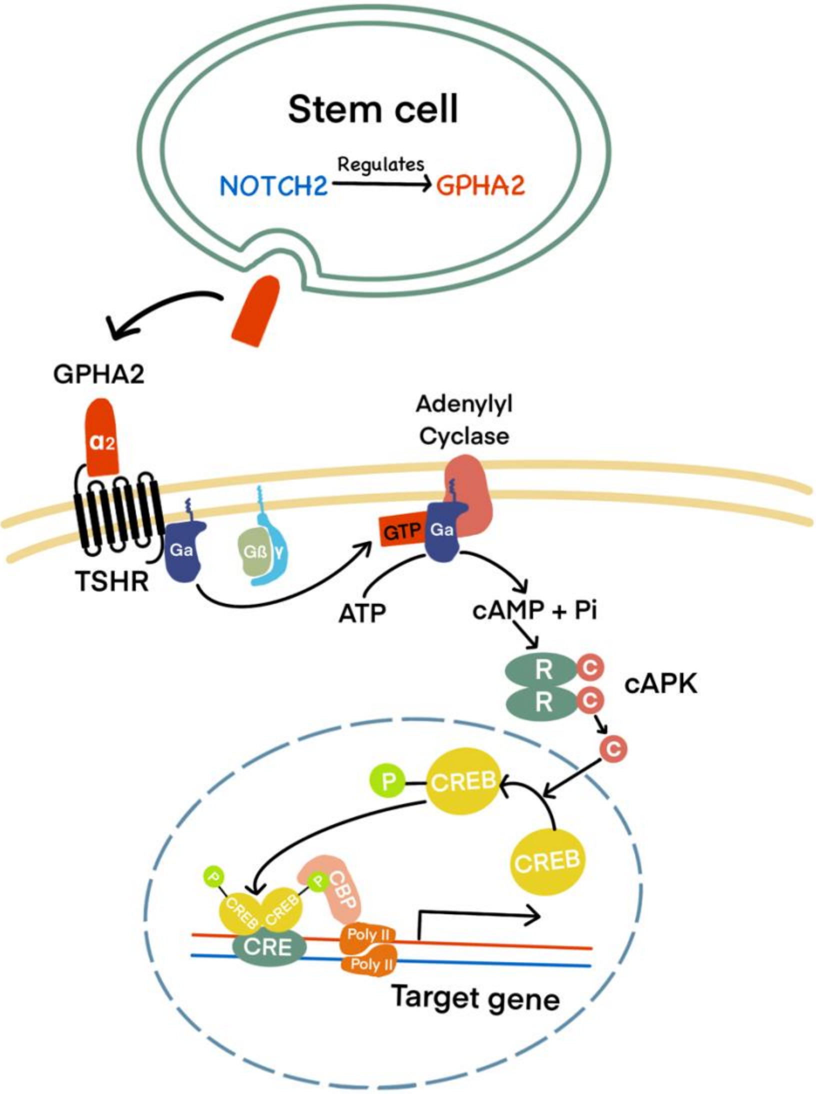

Figure 2.8 Hypothesized mechanisms of action of GPHA2 in the pituitary. Expression of *Gpha2* gene is regulated by *Notch2* in the pituitary stem cell, followed by secretion of GPHA2 peptide. GPHA2 acts through TSHR on neighboring cells and activated downstream signaling pathway.

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CHAPTER 3: PRENATAL EXPOSURE TO THE DEHP IMPACTS REPRODUCTION-RELATED GENE EXPRESSION IN THE PITUITARY[1]

Abstract

Phthalates are chemicals used in products including plastics, personal care products, and building materials, leading to widespread contact. Previous studies on prenatal exposure to Di-(2-ethylhexyl) phthalate (DEHP) in mice and humans demonstrated pubertal timing and reproductive performance could be affected in exposed offspring. However, the impacts at the pituitary, specifically regarding signaling pathways engaged and direct effects on the gonadotropins LH and FSH, are unknown. We hypothesized prenatal exposure to DEHP during a critical period of embryonic development (e15.5 to e18.5) will cause sex-specific disruptions in reproduction-related mRNA expression in offspring's pituitary due to interference with androgen and aryl hydrocarbon receptor (AhR) signaling. We found that prenatal DEHP exposure *in vivo* caused a significant increase in *Fshb* specifically in males, while the anti-androgen flutamide caused significant increases in both *Lhb* and *Fshb* in males. AhR target gene *Cyp1b1* was increased in both sexes in DEHP-exposed offspring. In embryonic pituitary cultures, the DEHP metabolite mono-(2-ethylhexyl) phthalate (MEHP) increased *Cyp1a1* and *Cyp1b1* mRNA in both sexes and *Cyp1b1* induction was reduced by co-treatment with AhR antagonist. AhR reporter assay in GHFT1 cells confirmed MEHP can activate AhR signaling. *Lhb*, *Fshb* and *Gnrhr* mRNA were significantly decreased in both sexes by MEHP, but co-treatment with AhR antagonist did not restore mRNA levels in pituitary culture. In summary, our data suggest phthalates can directly affect the function of the pituitary by activating AhR signaling and altering gonadotropin expression. This indicates DEHP's impacts on the pituitary could contribute to reproductive

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dysfunctions observed in exposed mice and humans.

Introduction

Di(2-ethyl-hexyl) phthalate (DEHP) is one of the most prevalent phthalates, used as a plasticizer and industrial solvent. DEHP can be found in various products including PVC materials, food containers, personal-care products, cosmetics, and medical devices. Humans and other animals are constantly exposed to DEHP on a daily basis and it can enter human bodies through different paths including ingestion, inhalation and dermal contact (Staples et al. 1997).

DEHP is defined as an endocrine disrupting chemical (EDC) due to its ability to cause endocrine dysfunction in humans and other animals (Adibi et al. 2003; Martinez-arguelles et al. 2013). Alteration in reproductive function is one of the most common effects of DEHP as an EDC (Kay et al. 2014; Zhou et al. 2017). In males, DEHP can have anti-androgenic effects and cause testicular-dysgenesis-like syndromes (TDS) including decreased anogenital distance (AGD), reduction in testosterone production, and alterations in sexual differentiation (Swan et al. 2005; Hu et al. 2009; Radke et al. 2018). These effects are consistent with effects of exposure to other anti-androgens such as flutamide (Kreisman, Song, Yip, B. V. Natale, et al. 2017), indicating that DEHP could affect androgen-mediated biological process. In females, DEHP can suppress estradiol production in the ovary (Lovekamp-Swan and Davis 2003) and accelerate reproductive aging (Hannon et al. 2016).

Humans and animals are constantly exposed to DEHP throughout life but the prenatal period is thought to be one of the critical windows during which DEHP exposure can cause more serious consequences. In male rodents, prenatal exposure to DEHP could affect spermatogenesis and sperm DNA methylation (Prados et al. 2015) and induce premature reproductive senescence, decreased fertility, decreased semen quality and mobility (Barakat et al. 2017); in females, the exposure could lead to abnormal follicular development (Zhou et al. 2017; Brehm et al. 2018), disrupted estrous cyclicity and folliculogenesis, early onset of puberty and decreased fertility-related indices (Niermann et al. 2015). Some effects can be passed on to the offspring in a transgenerational manner (Brehm et al. 2018). As for hormone changes, decreased Follicle Stimulating Hormone (FSH), Inhibin B and testosterone levels, as well as increased Luteinizing Hormone (LH) and estradiol levels were found in offspring (Brehm et al. 2018). Even though multiple findings suggest a correlation between prenatal DEHP exposure and reproductive function disruptions, the molecular mechanisms showing how DEHP exposure could cause those disruptions are unknown. Also, effective interventions that could reverse the negative effects of DEHP have not been found.

The reproductive system in humans and rodents is regulated by Hypothalamic-Pituitary-Gonadal (HPG) axis (Kaprara and Huhtaniemi 2018). Studies have found that prenatal exposure to DEHP could cause alterations in both the hypothalamus and pituitary. Specifically, *in utero* and lactational DEHP exposure in mice significantly increased gonadotropin gene expression in male and female offspring when examined in adulthood (Pocar et al. 2012). A similar exposure period in rats caused a decrease in estrogen receptor (ER)α and β expression in prepubertal and adult female pituitary (Pérez et al. 2020). Despite this mounting evidence showing that DEHP impacts HPG axis function during development, no studies have looked directly at the developing pituitary to determine direct effects, especially with lower doses of DEHP. Additionally, little is known about how DEHP could directly affect the pituitary gland through mechanisms other than its anti-androgenic effects.

Limited data suggest that DEHP, together with other groups of phthalates, can be weak agonists of aryl hydrocarbon receptor (AhR) and can activate AhR in multiple cell types of humans or rodents (Ernst et al. 2014). AhR is a transcription factor that is thought to function primarily as a sensor of environmental toxicants. AhR signaling activates the downstream transcription of genes including *Cyp1a1*, *Cyp1b1* and *Cyp1a2* (Formosa and Vassallo 2017) that are cytochrome P450s enzymes which are involved in the degradation, metabolism and excretion of toxicants. AhR signaling could be activated by a range of ligands in different categories including halogenated aromatic hydrocarbons (HAHs) like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), non-halogenated polycyclic aromatic hydrocarbons like β-naphthoflavone, and polychlorinated biphenyls (PCBs) (Petkov et al. 2010). DEHP or its primary metabolite- MEHP could induce AhR signaling *in vivo* in transgenic zebrafish (Junaid et al. 2018), as well as in mouse ovarian granulosa cells (Lovekamp-Swan et al. 2003). In *in vitro* experiments, DEHP modestly induced an AhR reporter luciferase expression vector in Hepa1.12cR cells (Krüger et al. 2008). However, there have not been any studies showing activation of AhR in the pituitary gland *in vivo* or *in vitro* by DEHP, and more specifically, how it could affect reproductive related gene expression.

It is known that AhR signaling impacts the pituitary gland. The AhR signaling pathway is crucial for maintaining the normal reproductive functions of the pituitary gland, as gonadotropin mRNA expression in the pituitary was significantly decreased in AhRKO mice (Hattori et al. 2018). Other than its endogenous functions, AhR activation with TCDD has been known to be able to decrease *Lhb* expression in both male and female rodents (Mutoh et al. 2006), and activation with agonist β-naphthoflavone was found to reduce the expression of *Ahr* and *Prl* mRNA in GH3 cells (Moran et al. 2012). There are also contradictory findings in ovariectomized female mice- an increase in *Cga*, *Lhb* and *Esr1* mRNA levels were caused by TCDD exposure (Cao et al. 2011). Although plenty of studies showed the impacts of AhR activation on the reproductive-related functions in the pituitary, most of them only focused on traditional AhR agonists such as TCDD or β-naphthoflavone. Little is known about the effects of other possible AhR agonists, like DEHP, on the pituitary.

In this study we aimed to 1) determine the effects of developmental exposure to DEHP on pituitary gonadotropes and its relationship to androgen signaling; 2) elucidate the direct effects of DEHP and its primary metabolite, MEHP, on the pituitary, and examine the impact of DEHP on AhR signaling and the possible relationship between AhR and gonadotropin regulation. We performed dosing experiments, embryonic pituitary explant culture and dual-luciferase reporter assay, which provided us both *in vivo* and *in vitro* evidence to explore DEHP's impacts on reproductive function.

Materials and Methods

Mice

CD-1 mice were originally obtained from Charles River and were bred in house at the animal facility of the University of Illinois Urbana-Champaign for all experiments described. Sex was confirmed by visual inspection and SRY genotyping of the tail using the primer sequences listed in table 1. The University of Illinois Urbana-Champaign Institutional Animal Care and Use Committee approved all procedures.

In vivo dosing and pituitary collection

Timed-pregnant CD-1 mice were randomly divided into 3 different dosing groups: control, flutamide and DEHP group, each group contains 5-7 dams. Starting from gestational day 15.5 until 18.5, each mouse received a daily dose orally through consumption of chemicals dissolved in tocopherol stripped corn oil from a pipet tip at the same time every day. The time period e15.5-e18.5 was selected because male mice have a prenatal testosterone surge which is critical for gonad and sexually dimorphic development (Weisz and Ward 1980). This testosterone surge starts at embryonic day 15.5 and studies have shown that blocking this testosterone surge by exposing males prenatally with anti-androgens could affect gonadotropin expression in males and make them female-like (Kreisman, Song, Yip, B. V. Natale, et al. 2017). The doses in flutamide (Tocris) and DEHP (Sigma) group were 10 mg/day and 200 µg/kg/day, respectively; the control group received 10 µL DMSO. The dosage of flutamide was picked based on a previous paper showing effects on pituitary gonadotropins (Kreisman, Song, Yip, B. V. Natale, et al. 2017). The dosage of DEHP, 200 µg/kg/day, was chosen because it is the dosage that is closest to human daily exposure range (DEHP TDI: ~50µg/kg/day) and also had reproductive outcomes in both male and female offspring (Niermann et al. 2015). The whole pituitary gland was taken out from offspring on the day of birth (P0) and preserved in RNAlater (Thermo-Fisher) at -20 °C.

Anogenital distance and weight measuring

Anogenital distance (AGD) and body weight were measured using a caliper and an electronic scale, respectively. The AGDs were normalized to body weight for analysis.

In vitro pituitary culture

Embryos from CD-1 mice were taken out of the uterus on embryonic day 16.5 and tissue from each embryo was collected for SRY genotyping for sex determination. The whole pituitary glands were taken out and transferred to 96-well plate with 50µL phosphate buffered saline (PBS) +1% antibiotic/antimycotic solution (anti-anti, Thermo-Fisher). Pituitaries were washed twice with PBS +1% anti-anti and 50 µL of media (phenol red-free DMEM/F12 (Thermo-Fisher), supplemented with 10% charcoal-stripped fetal bovine serum (Sigma-Aldrich) and 1% anti-anti) with different treatments (0.1 µg/mL, 1 µg/mL, 10 µg/mL and 100 µg/mL MEHP/DEHP (Sigma) added to each well. The concentrations of MEHP/DEHP were picked based on previous papers describing mouse ovarian cultures with MEHP/DEHP (Hannon, Brannick, Wang, and Flaws 2015; Hannon, Brannick, Wang, Gupta, et al. 2015). Pituitaries were cultured at 37 °C for 72 hours without changing the media. To co-treat the pituitary with MEHP and AhR antagonist- CH223191 (ApexBio), pituitaries were harvested and washed as described above and first treated with the antagonist dissolved in DMSO in the 37 °C incubator for 2 hours, then MEHP was added at the final concentration of 100 µg/mL. Some pituitaries were only treated with the antagonist to eliminate possible effects of the antagonist. The final concentration of the AhR antagonist was at 0.33 µg/mL as instructed by the manufacturer’s product catalog.

RNA preparation and cDNA preparation

Pituitaries from both the *in vivo* and *in vitro* experiments were homogenized and RNA was isolated using the RNAqueous-Micro kit (Invitrogen). Total RNA was reverse-transcribed according to the manufacturer's instructions using the ProtoScript Strand cDNA Synthesis kit (New England Biolabs) as previously described (Nantie et al. 2014).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Pituitaries from in vivo experiments (n = 5-7) or culture from in vitro experiments (n = 6-22), were subjected to qPCR for mRNA analysis of specific genes. Oligonucleotide primers for *Ahr, Gnrhr*, *Lhb*, *Fshb*, *Prl*, *Cyp1a1* and *Cyp1b1* (Life Technologies, primer sequence see Table 1) were used to amplify gene-specific transcripts by qPCR. The expression levels of genes of interest were normalized to *Ppia* mRNA levels. *Ppia* is a stable housekeeping gene for qRT-PCR normalization(Muñoz et al. 2021) that we confirmed had consistent expression levels across different treatments both *in vivo* and *in vitro*. The data were analyzed using the standard comparative cycle threshold value method ($2^{-\Delta\Delta Ct}$) (Goldberg et al. 2011).

Table 3.1: PCR primers used in this study

Gene	Forward Sequence 5'-3'	Reverse sequence 3'-5'
Ppia	CAAATGCTGGACCAAACACAAACG	GTTCATGCCTTCTTTCACCTTCCC
Sry	TGCAGCTCTACTCCAGTCTTG	GATCTTCATTTTTAGTGTTC
Cyp1a1	GTCCCGGATGTGGCCCTTCTCAAA	TAACTCTTCCCTGGATGCCTTCAA
Cyp1b1	AATGAGGAGTTCGGGCGCACA	GGCGTGTGGAATGGTGACAGG
Fshb	TGGTGTGCGGGCTACTGCTAC	ACAGCCAGGCAATCTTACGGTCTC
Gnrhr	ATGATGGTGGTGATTAGCC	ATTGCGAGAAGACTGTGG
Lhb	CCCAGTCTGCATCACCTTCAC	GAGGCACAGGAGGCAAAGC

Transfection and dual-luciferase reporter assay

Immortalized pituitary progenitor cell line GHFT1 cells (Lew et al. 1993)(kindly used with permission from Dr. Pamela Mellon) were maintained in DMEM media with phenol red (Thermo-Fisher), supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin (Fisher). Cells were plated in 24-well plate and transfected at ~70% confluency. 500 ng AhR reporter construct (Cignal) mixed with 1:40 renilla control reporter construct (Cignal) were transfected in each well with 1.5 μL Lipofectamine 2000 (Invitrogen) as transfection reagent and Opti-MEM (Thermo-Fisher) as transfection media. After 6 hours of transfection, media was replaced and fresh phenol red-free DMEM/F12 media (Thermo-Fisher), supplemented with 10% charcoal-stripped fetal bovine serum (Sigma-Aldrich) and 1% anti-anti with different treatments was added. Treatments consisted of 100 μg/mL MEHP, 3.22 ng/mL TCDD or DMSO as the control treatment. After 12-hour treatment, cells were lysed and dual-luciferase assay system (Promega) was performed according to manufacturer's instructions. Relative luciferase units (RLU) were detected using a luminometer (High-Throughput Screening Facility, UIUC) and normalized to renilla RLU.

Statistical analysis

For *in vivo* experiments, male and female offspring were analyzed separately and data from multiple pups in one litter were averaged to one data point and each litter was considered as one unit statistically. For *in vitro* experiments, each pituitary in one culture was considered as one data point and at least three culture experiments were performed.

All data are presented as mean + / - SEM. Statistical significance was determined using two-way (*in vivo*) or one-way (*in vitro*) ANOVA followed by Tukey's multiple comparisons test or Dunnett's multiple comparisons test. P values less than 0.05 were considered significant. All analyses were performed using Graph Pad Prism 8.2.1.

Results

Gonadotropin gene expression is affected by DEHP or MEHP in the developing pituitary

To determine if DEHP interferes with testosterone signaling, we focused dosing on the period of testosterone surge in male mice (e15.5 to e18.5) and collected offspring at PND 0. We also used a known anti-androgen, flutamide, to compare to the effects of DEHP.

We measured the anogenital distance (AGD) of the offspring (Fig 3.1) right before collecting the pituitaries because AGD is reflective of testosterone levels. We found no significant change in AGD normalized to body weight in both male and female offspring exposed to DEHP. However, as expected, male offspring treated with flutamide had a significant decrease in AGD/BW compared to control group.

We then looked at mRNA expression changes of gonadotropin and related genes in the offspring and found DEHP exposure significantly increased the mRNA level of follicle stimulating hormone subunit beta (*Fshb*) in males only (Fig 3.2 B). However, the induction was not observed for luteinizing hormone subunit beta (*Lhb*) in either sex (Fig 3.2 A). Flutamide successfully increased expression levels of both *Lhb* and *Fshb* in males, and as expected, it had no effect on females due to female's lack of prenatal androgen surge (Fig 3.2 A-B). We also examined the expression of gonadotropin releasing hormone receptor (*Gnrhr*) mRNA and found no significant change caused by either DEHP or flutamide in both sexes (Fig 3.2 C).

To further explore whether DEHP could directly cause those changes described above at the level of the pituitary, we conducted *in vitro* embryonic pituitary culture experiments and checked gonadotropins and related gene expression changes in cultured pituitaries. Cultures were treated with DEHP's primary metabolite MEHP because there is a lack of metabolic machinery to convert DEHP to MEHP in the *in vitro* setting (Hannon, Brannick, Wang, and Flaws 2015). In contrast to *in vivo* experiments, both *Lhb* (Fig 3.3 A-B) and *Fshb* (Fig 3.3 C-D) mRNA levels were significantly decreased when treated with 100 µg/mL MEHP in both male (Fig 3.3 A, C) and female (Fig 3.3 B, D) pituitaries. As for *Gnrhr* mRNA, there is no significant difference caused by MEHP treatment only with a trend of decrease at the highest concentration (100 µg/mL). Taken together, these data demonstrate that both *in vivo* and directly at the embryonic pituitary, DEHP can affect gonadotropin mRNA production.

AhR downstream genes were induced with both in vivo and in vitro exposure to DEHP or MEHP

To determine if DEHP engaged AhR signaling in the pituitary, we looked at mRNA levels of *Cyp1a1* and *Cyp1b1*, two genes that are transcriptionally increased by AhR signaling. We found no change in *Cyp1a1* (Fig 3.4 A) mRNA levels but a significant increase in *Cyp1b1* (Fig 3.4 B) mRNA in both sexes to similar levels. Interestingly, flutamide had no effect on both genes in either sex. For the *in vitro* culture experiment, since we found no baseline difference between male and female *Cyp1a1* or *Cyp1b1* genes, we combined the data of both sexes when analyzing. Both 0.1 and 100 µg/mL treatment of MEHP caused a significant increase in *Cyp1a1* mRNA level (Fig 3.5 A), while *Cyp1b1* mRNA level was significantly increased by 10 and 100 µg/mL treatment (Fig 3.5 B). We also treated the pituitaries with DEHP and examined the expression change of both genes and found no change caused by DEHP at the two higher concentrations (Fig 3.5 C-D), which confirmed that *in vitro*, MEHP was the chemical that exerted the effects directly at the pituitary. In all, these data show that DEHP/MEHP can likely signal through AhR in the embryonic pituitary.

To confirm whether the induction of *Cyp1a1* and *Cyp1b1* mRNA by MEHP occurs through the AhR signal pathway, we co-treated the pituitary explants with MEHP and AhR antagonist-CH223191 and checked whether the induction could be reversed (Fig 3.6). The induction of *Cyp1a1* mRNA was not blocked by the antagonist as no significant difference between the MEHP and MEHP + antagonist treatment group was found, and both are significantly increased compared to control group (Fig 3.6 A). In addition, the treatment of AhR antagonist alone significantly decreased *Cyp1a1* expression. As for *Cyp1b1* (Fig 3.6 B), the co-treatment of AhR antagonist with

MEHP successfully brought the mRNA level back to the level of control. Interestingly, the antagonist-alone treatment did not affect *Cyp1b1* mRNA. Taken together, these studies demonstrate that the induction of *Cyp1b1* by MEHP was indeed mediated through activation of AhR signaling.

MEHP directly activates AhR as shown by AhR reporter assay

To further determine whether MEHP could directly activate AhR signaling, we performed an AhR reporter assay with a reporter construct containing xenobiotic response element (XRE) linked to luciferase in the immortalized pituitary progenitor cell line GHFT1. We observed MEHP treatment at the concentration of 100 mg/mL significantly induced the luciferase activity compared to DMSO treatment as control. We also treated the cells with TCDD as positive control to guarantee a functional AhR reporter construct (Fig 3.7). These data confirmed MEHP could activate AhR signaling in the pituitary and induce transcription through XRE.

The decrease of gonadotropin genes in vitro was not directly caused by activation of AhR signaling

Next, we examined the expression change of gonadotropins and related genes when co-treated with AhR antagonist in order to find out whether there is direct association between the activation of AhR signaling and decrease in gene expression (Fig 3.8). For all three genes- *Lhb* (Fig 3.8 A-B), *Fshb* (Fig 3.8 C-D) and *Gnrhr* (Fig 3.8 E-F), co-treatment with AhR antagonist did not bring the mRNA back to control levels; there is no significant difference between MEHP alone and MEHP + antagonist treatment group. This suggests that even though the AhR was activated by MEHP, and MEHP caused the decrease of gonadotropin genes at the level of pituitary, there is no direct association between these two effects.

Discussions

DEHP has anti-androgenic effects on male rodents through possible molecular mechanisms including interfering with cholesterol transport and steroidogenesis in the testis (Borch et al. 2006). In this study we investigated possible anti-androgenic effects of DEHP on prenatally exposed offspring, specifically focusing on impacts at the pituitary and reproductive gene expression. Compared to the potent anti-androgen, flutamide, DEHP at the dose of 200 μg/kg/day did not significantly affect ADG- a toxicological marker for fetal androgen action (Schwartz et al. 2019) of the offspring. However, this could be due to the relatively lower dosage and the shorter dosing period (e15.5 through e18.5) we chose, or could also be due to the age that we chose to measure for the offspring (PND 0) since previous studies showed a higher dosage (750 mg/kg/day) with longer dosing period (e10.5 through e18.5) of DEHP could decrease the male AGD at the ages of PND 21 and 16 months (Barakat et al. 2017). Another difference may be mechanism of action. DEHP acts by interfering with the regulation of steroidogenesis (J et al. 2006) while flutamide acts as an androgen receptor antagonist (Simard et al. 1986).

Even though no significant anti-androgenic impact of DEHP on prenatal exposed offspring was found at the physiological level, evidence indicating that DEHP could impact androgen signaling was found at the molecular level- a significant increase of *Fshb* mRNA level was found only in male offspring exposed to DEHP. This change of *Fshb* caused by DEHP was consistent with the impact of flutamide and is similar to another study showing exposure to flutamide during the testosterone surge increases *Fshb* in males (Kreisman, Song, Yip, B. V. Natale, et al. 2017).

Interestingly, *Lhb* was not affected by DEHP, which is often thought to be correlated with *Fshb* and they were expected to change in a similar way as they are both regulated by steroid hormones in a negative-feedback loop. This indicates that more than one mechanism could be involved in regulating these two gonadotropin genes in response to DEHP which caused them to change differently. Transcription of both *Lhb* and *Fshb* could be regulated by GnRH pulsatility- higher pulse frequency favors *Lhb* while lower frequency favors *Fshb* (Thompson and Kaiser

2014). Future studies should examine GnRH pulsatility in detail and explore whether DEHP exposure is associated with GnRH pulsatility changes. Besides, transcription of *Fshb* could also be regulated independently by an activin-follistatin-inhibin loop: activin released by gonads upregulates *Fshb* while inhibin and follistatin downregulate it (Gregory and Kaiser 2004). In a previous study, it has been found that prepubertal male offspring exposed to DEHP during gestation and lactation had significantly decreased serum inhibin B (Borch et al. 2004; Brehm et al. 2018), and this could be a possible explanation for the increase of *Fshb* found in our study. Future experiments should examine possible changes of activin, inhibin and follistatin after DEHP exposure to better understand the *in vivo* mechanisms regulating *Fshb*'s expression change caused by DEHP.

We saw a decrease in both *Lhb* and *Fshb* mRNA in the pituitary explant culture experiment treated with 100 µg/mL MEHP. These opposite results compared to *in vivo* data indicate that different mechanisms regulating the expression of gonadotropins other than androgen signaling and the negative feedback loop through HPG axis could be involved when the body is exposed to DEHP/MEHP. One possible mechanism that we considered was the AhR signaling pathway since it is related to metabolism of toxicants in the body and it is also active in the pituitary (Formosa and Vassallo 2017; Tapella et al. 2017). DEHP has been known as a weak agonist of AhR (Ernst et al. 2014) and activation of AhR by DEHP has been found in different organisms (Ernst et al. 2014; Tsai et al. 2014; Junaid et al. 2018). Specifically, when it comes to AhR activation and gonadotropin regulation, decreased *Lhb* and *Fshb* mRNA level and serum level of LH and FSH were observed in mice with the AhR signaling pathway activated developmentally (Mutoh et al. 2006; Hattori et al. 2018). These findings are consistent with our *in vitro* culture experiment results but are not reflected by our *in vivo* experiments. This suggests that in contrast to DEHP/MEHP's antiandrogenic effects that are likely exerted through the HPG axis, activation of the AhR signaling pathway by DEHP/MEHP directly at the pituitary is associated with decreased gonadotropin gene expression.

We demonstrated induction of AhR downstream genes *Cyp1a1* and *Cyp1b1* by MEHP *in vitro*, however, we observed a more complicated expression change of these two genes *in vivo*. Both male and female offspring from the *in vivo* experiment had an increase in *Cyp1b1* mRNA level after being exposed to DEHP prenatally, while the *Cyp1a1* remained unchanged. The induction of *Cyp1b1* mRNA was consistent with our hypothesis that AhR was activated by DEHP. However, a possible explanation for upregulated *Cyp1b1* and unchanged *Cyp1a1 in vivo* is that there might be other mechanisms involved that downregulated *Cyp1a1*, which counteracted the induction of *Cyp1a1* by AhR activation after DEHP exposure. It has been known that 17β-estradiol could reduce only *Cyp1a1* but not *Cyp1b1* expression at the transcriptional level by squelching available nuclear factor-1 in human endometrial cells (Ricci et al. 1999). Future studies could focus more specifically on the relationship between DEHP activation and its effects on estrogen and estrogen receptor regulation and possible association with AhR or other signaling pathways. Even though *in vivo* experiments showed only *Cyp1b1* mRNA was induced by DEHP exposure, both *Cyp1a1* and *Cyp1b1* mRNA were found increased by MEHP in the *in vitro* culture experiment. Interestingly, we saw a nonmonotonic dose-response curve in *Cyp1a1* mRNA levels, marked by higher level of *Cyp1a1* expression in pituitaries treated with 0.1 µg/mL MEHP compared to 1 and 10 µg/mL MEHP. Nonmonotonic dose effects are often observed in response to EDC exposures (Vandenberg et al. 2012). Further, prenatal exposure to DEHP has been reported to have nonmonotonic dose effects on testicular and serum testosterone and AGD in male mice (Do et al. 2012). Although the mechanisms causing the nonmonotonic dose effects are still unknown, our findings showing nonmonotonic dose effects on *Cyp1a1* in response to MEHP treatment could be crucial for future studies on the effects of exposure to phthalates, especially environmentally relevant low-dose exposures. Besides, the absence of nonmonotonic dose effects on *Cyp1b1* is another indication that multiple mechanisms could be involved in regulating *Cyp1a1* and *Cyp1b1* expression in the pituitary in response to DEHP/MEHP exposure. These findings above confirmed that MEHP could directly impact the pituitary and the comparison to DEHP treatment showed that at the pituitary, DEHP could only exert its effects after being metabolized to MEHP. The different changes of *Cyp1a1 in vitro* compared to *in vivo* also indicated that multiple mechanisms are likely to be involved when the body was exposed to DEHP and the effects through other mechanisms could be opposite to the direct effects at the pituitary.

We discovered the induction of *Cyp1b1*, but not *Cyp1a1*, by MEHP could be blocked by cotreatment with an AhR antagonist. One possible explanation for the antagonist not being able to block the *Cyp1a1* induction by MEHP was that the induction might be mediated through the combination of AhR and other signaling pathways. One candidate that could be considered is the peroxisome proliferator-activated receptors (PPARs), which are ligand activated nuclear receptors that function as transcription factors to regulate gene expression in response to endogenous and exogenous ligands (Peraza et al. 2006). MEHP/DEHP is considered a ligand of PPARs and it has been known to be able to disrupt male and female reproductive tract through PPARs (Latini et al. 2008). PPARs have been reported to be expressed in the pituitary (Heaney et al. 2003; Bogazzi et al. 2005; Knig et al. 2009) and could induce *Cyp1a1* expression independent from AhR pathway (Sérée et al. 2004). Thus, PPAR signaling pathway could be one of the directions to investigate to understand the induction of *Cyp1a1* and DEHP exposure for future study. It is also possible that the AhR antagonist could have ligand or target gene selective effects. For example, CH223191 was reported to have preferential inhibitory effects on HAHs-type ligands like TCDD, but have little effect on BNF and PAHs (Zhao et al. 2010). Another interesting finding was that the AhR antagonist alone was able to decrease *Cyp1a1* mRNA expression in the absence of MEHP. This suggests that the basal level of *Cyp1a1* expression could be decreased by the presence of AhR antagonist and the unchanged *Cyp1a1* in response to AhR antagonist indicated the possible involvement of different mechanisms in regulation of *Cyp1a1* and *Cyp1b1*.

Because we saw an inconsistent response of *Cyp1a1* and *Cyp1b1* to AhR antagonist, we further explored the activation of AhR by MEHP using an AhR reporter assay. We demonstrated MEHP directly activates AhR signaling in the pituitary by showing significant increases in normalized luciferase activity in GHFT1 cells treated with MEHP. The induction was not as strong as that elicited by TCDD, suggesting DEHP/MEHP have a reduced ability to induce AhR signaling compared to the prototypical ligand. Taken together, these data suggest that DEHP/MEHP can contribute to regulation of AhR target genes, although they make act as selective receptor modulators on specific genes such that *Cyp1b1* is more responsive than *Cyp1a1*.

To determine whether the decrease of gonadotropins and *Gnrhr* was directly associated with the activation of AhR, we examined the mRNA levels of *Lhb*, *Fshb*, and *Gnrhr* in pituitaries co-treated with MEHP and AhR antagonist and found the co-treatment with antagonist did not restore the expression levels of these three genes. This finding showed us that the activation of AhR was not the direct cause of decrease in gonadotropin-related genes and also suggested that alternative pathways need to be discovered to determine the molecular mechanisms regulating the gonadotropin-related gene expression at the pituitary. PPARs mentioned earlier are one of the possible mechanisms since a previous study reported that PPAR agonists could directly suppress the gonadotropin transcription in mouse gonadotrope LbT2 cells (Takeda et al. 2007). Besides, DEHP was also reported to be related to immune responses (Koike et al. 2009; Kitaoka et al. 2013) and oxidative stress (Kasahara et al. 2002) and these factors could potentially play roles in the regulation of gonadotropins. In order to understand those possible mechanisms, future studies on the immune response and oxidative stress signaling specifically at the pituitary in response to DEHP exposure are needed.

In summary, our study showed that DEHP/MEHP could directly impact gonadotropin gene expression at the pituitary, and multiple mechanisms are likely involved in the regulation of gonadotropin genes in response to DEHP/MEHP exposure since different results were found from *in vivo* and *in vitro* experiments. AhR signaling pathway is one of the mechanisms that could be the mediator of gonadotropin regulation, however, no direct association was found between the AhR activation and decrease in gonadotropins. These results suggest the need for more extensive research on DEHP/MEHP's impact at the pituitary associated gonadotropin regulation.

Acknowledgements

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Figures

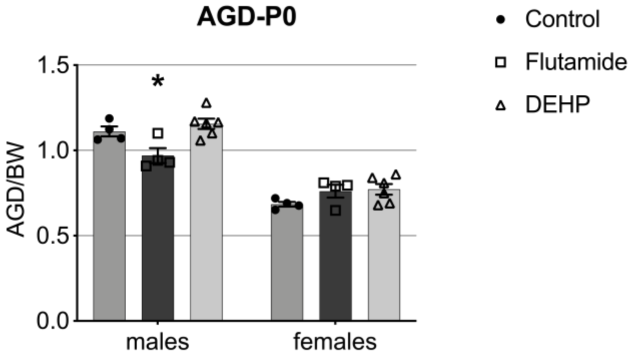

Figure 3.1 Anogenital distance (AGD) normalized to body weight (BW) of offspring at P0. Different columns refer to control, flutamide and DEHP treatment, respectively. AGD/BW was calculated, and the graph represents the mean +/− SEM (n = 4-6/treatment group). Two-way ANOVA followed by Tukey's multiple comparisons test was done. Asterisk (*) represents significant difference from vehicle control, Two-way ANOVA P = 0.0155, *P<0.05 by Tukey's multiple comparisons test.

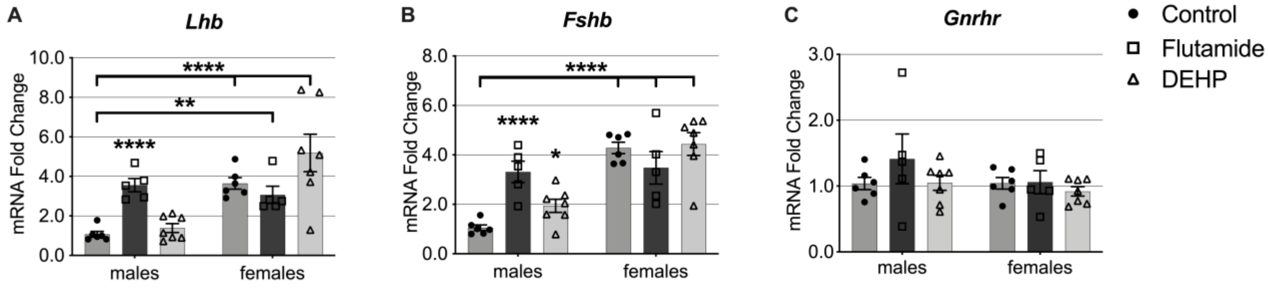

Figure 3.2 Pituitary gene expression changes in gonadotropes of offspring in response to DEHP exposure. mRNA fold changes of *Lhb* (A), *Fshb* (B) and Gnrhr (C) are graphed relative to male controls. Different columns refer to control, flutamide and DEHP treatment, respectively. The graph represents the mean +/− SEM (n = 5-7/treatment group). Two-way ANOVA followed by Tukey's multiple comparisons test was done. Asterisk (*) represents significant difference from male vehicle control, Two-way ANOVA P = 0.0172 (A), 0.0158 (B), *P<0.05, ****P<0.005, ****P<0.0001 by Tukey's multiple comparisons test

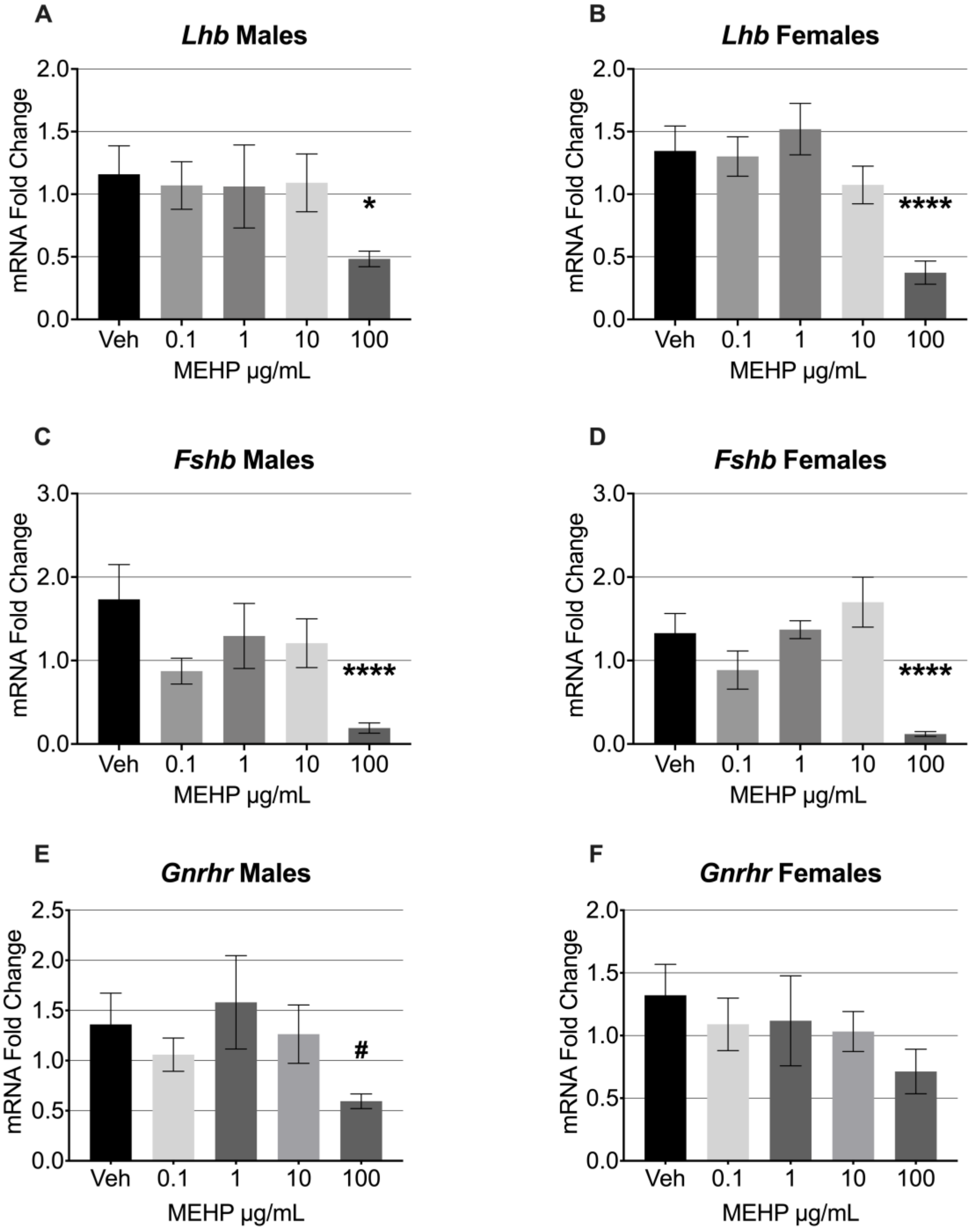

Figure 3.3 Pituitary gene expression changes in gonadotropes from in vitro culture experiment. mRNA fold changes of *Lhb* (A-B), *Fshb* (C-D) and *Gnrhr* (E-F) are presented with males and females separated. Different columns refer to concentration of MEHP as marked. The graph represents the mean +/− SEM (n = 5-12/treatment group). One-way ANOVA followed by Dunnett's multiple comparisons test was done. One-way ANOVA P = 0.0397 (A), P < 0.0001 (B-D), #: P = 0.053 (E). Asterisk (*) represents significant difference from vehicle control, *P<0.05, ****P<0.0001 by Dunnett's multiple comparisons test

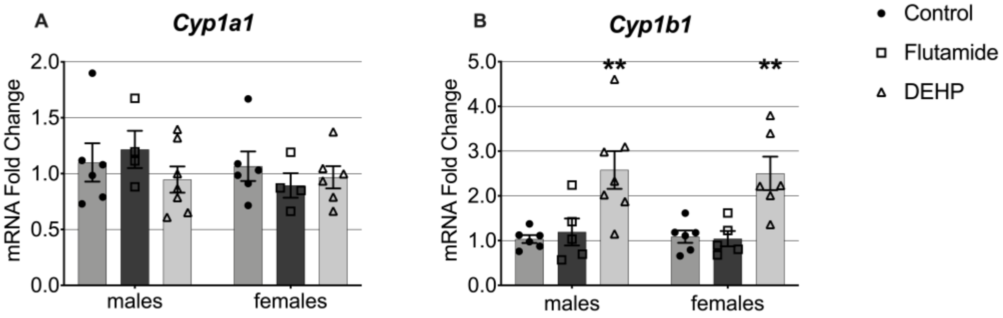

Figure 3.4 Pituitary gene expression changes of AhR downstream genes in offspring. mRNA fold changes of *Cyp1a1* (A) and *Cyp1b1* (B) are presented with males and females separated. Different columns refer to control, flutamide and DEHP treatment, respectively. The graph represents the mean +/− SEM (n = 5-7/treatment group). Two-way ANOVA followed by Tukey's multiple comparisons test was done. Asterisk (*) represents significant difference from vehicle control, Two-way ANOVA P < 0.0001 (B), **P<0.005 by Tukey's multiple comparisons test

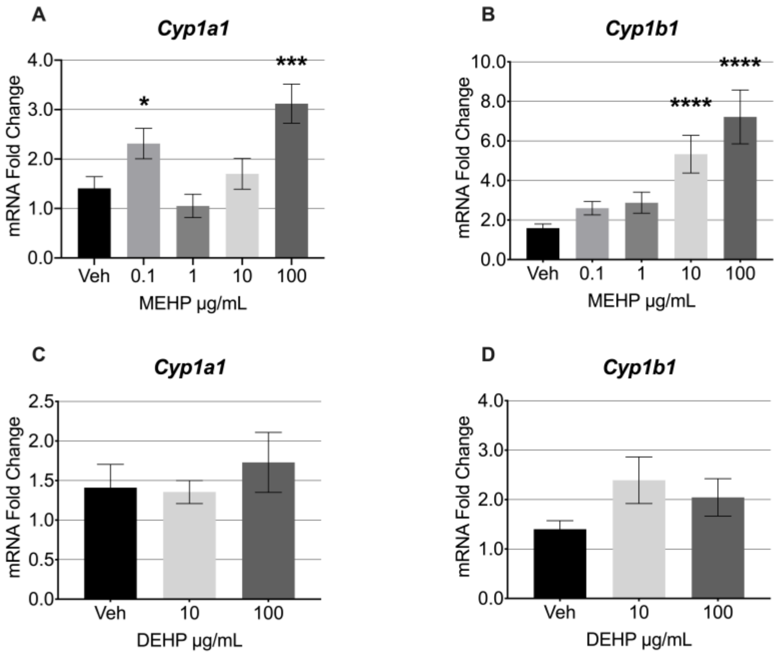

Figure 3.5 Pituitary gene expression changes of AhR downstream genes from in vitro culture experiment. mRNA fold changes of *Cyp1a1* (A, C) and *Cyp1b1* (B, D) are presented with males and females combined. Different columns refer to treatment of MEHP (A-B) and DEHP (C-D) as marked. The graph represents the mean +/− SEM (n = 8-22/treatment group in A and B, n = 4-8/treatment group in C and D). One-way ANOVA followed by Dunnett's multiple comparisons test was done. One-way ANOVA P <0.0001 (A-B). Asterisk (*) represents significant difference from vehicle control, *P<0.05, ***P<0.0005, ****P<0.0001 by Dunnet's multiple comparisons test

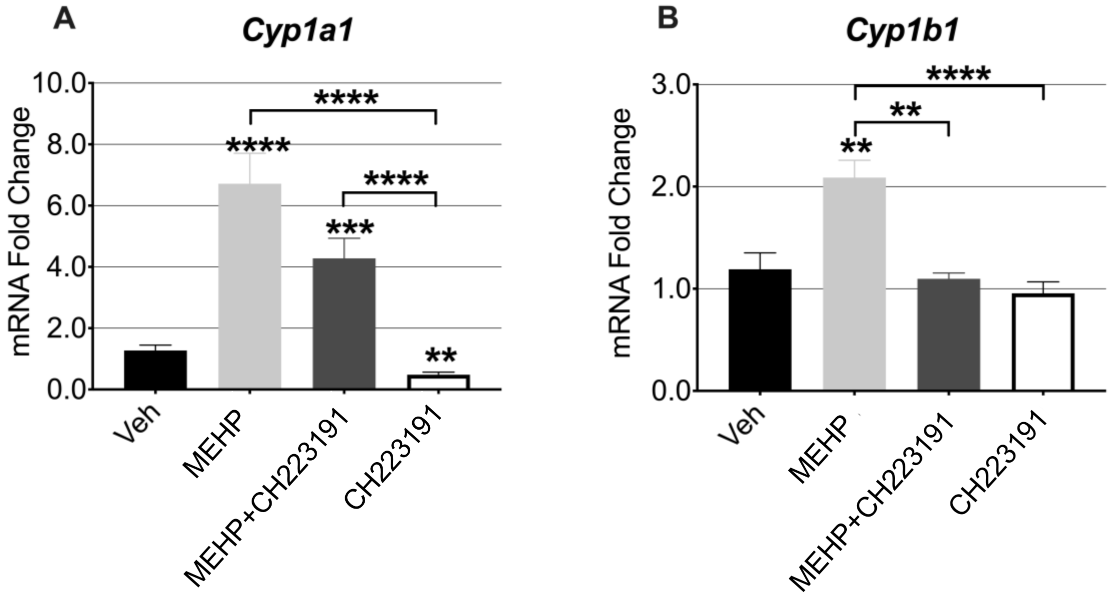

Figure 3.6 Pituitary gene expression changes of AhR downstream genes from in vitro culture experiment co-treated with AhR antagonist. mRNA fold changes of *Cyp1a1* (A) and *Cyp1b1* (B) are presented with males and females combined. Different columns refer to treatment of MEHP (100 μg/mL) and/or AhR antagonist CH223191 (0.33 μg/mL) as marked. The graph represents the mean +/− SEM (n = 9-10/treatment group). One-way ANOVA followed by Tukey's multiple comparisons test was done. One-way ANOVA $P < 0.0001$. Asterisk (*) represents significant difference from vehicle control or from other treatment group as indicated. *$P<0.05$, **$P<0.005$, ***$P<0.0005$, ****$P<0.0001$ by Tukey's multiple comparisons test.

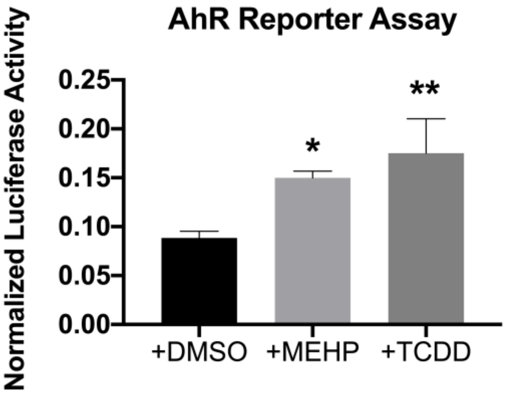

Figure 3.7 AhR reporter assay. Normalized luciferase activity in cells treated with 100 μg/mL MEHP or 3.22 ng/mL TCDD were compared to DMSO as the control treatment. The graph represents the mean +/− SEM (n = 4-6/treatment group). One-way ANOVA followed by Dunnett's multiple comparisons test was done. One-way ANOVA $P = 0.0066$. Asterisk (*) represents significant difference from DMSO control *$P<0.05$, **$P<0.005$ by Dunnett's multiple comparisons test.

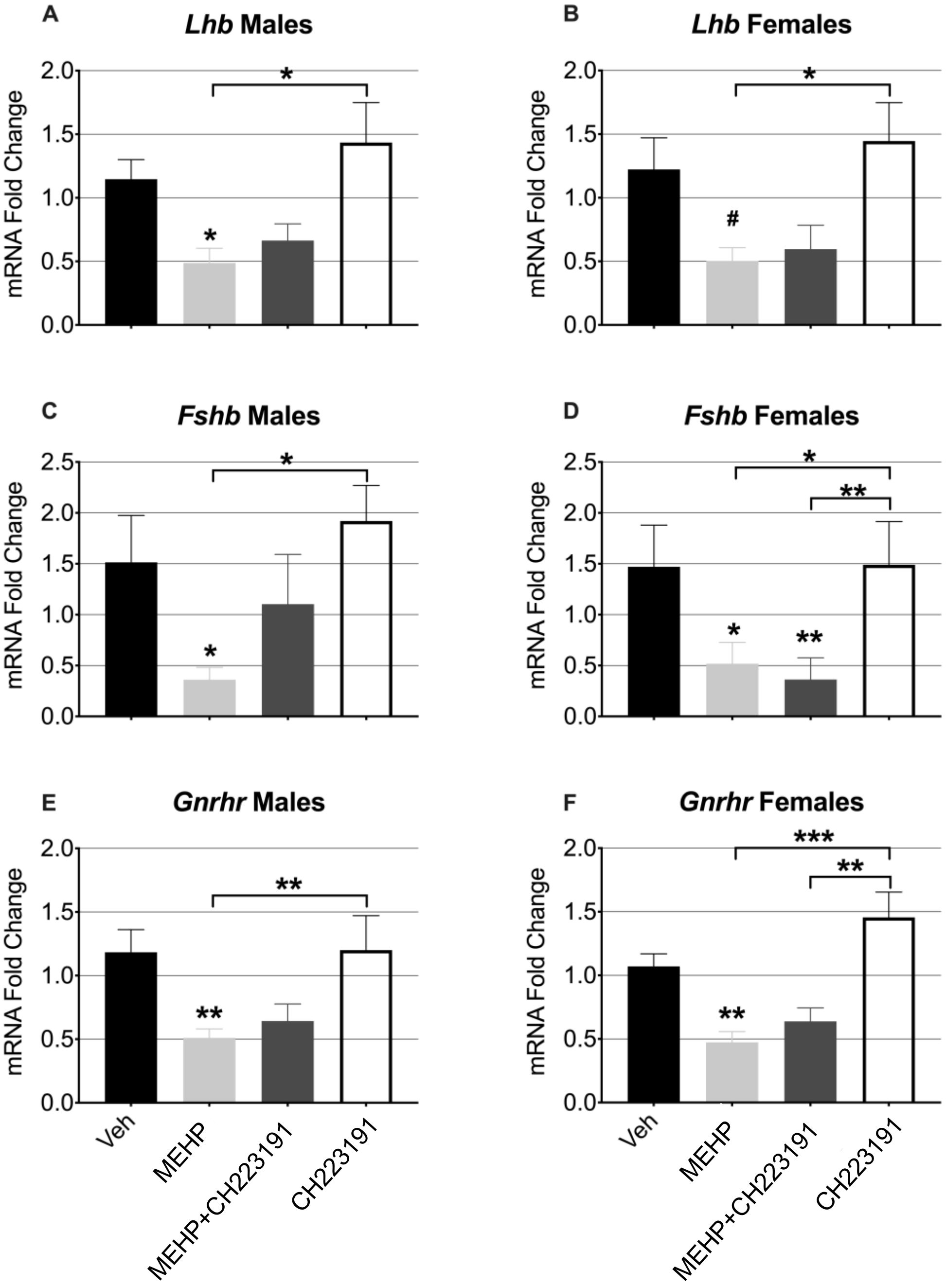

Figure 3.8 Pituitary gene expression changes of gonadotropes from in vitro culture experiment co-treated with AhR antagonist. mRNA fold changes of *Lhb* (A-B), *Fshb* (C-D) and Gnrhr (E-F) are presented with males and females separated. Different columns refer to treatment of MEHP (100 µg/mL) and/or AhR antagonist CH223191 (0.33 µg/mL) as marked. The graph represents the mean +/− SEM (n = 6-8/treatment group). One-way ANOVA followed by Tukey's multiple comparisons test was done. One-way ANOVA P = 0.0054 (A), 0.0134 (B), 0.0067 (C), 0.0005 (D), 0.0017 (E), 0.0001 (F). Asterisk (*) represents significant difference from vehicle control or from other treatment group as indicated. *P<0.05, **P<0.005, ***P<0.0005. #: Control vs. 100 µg/mL MEHP, P=0.067 by Tukey's multiple comparisons test.

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CHAPTER 4: EFFECTS OF ENVIRONMENTALLY RELEVANT PHTHALATE MIXTURE ON THE PITUITARY GLAND

Abstract

Phthalates are a group of endocrine disrupting chemicals used as solvents and plasticizers that are prevalent in daily life. Constant exposure to phthalates in humans could cause dysfunctions in multiple systems and reproductive dysfunctions are the most common outcomes of phthalate exposure. Embryonic development is a particularly sensitive period to the impacts of phthalate exposure, which can lead to long term consequences. Most studies on phthalates' impacts focused on single phthalate, however, urine sample analysis revealed that humans are exposed to a mixture of different types of phthalates and it is critical to understand the effects of phthalate mixture comparing with a single phthalate. In this study we investigated the impacts of prenatal exposure to an environmentally relevant phthalate mixture on the reproductive functions of the offspring, specifically at the level of the pituitary, an understudied part of the reproductive axis. We found that prenatal phthalate mixture exposure caused no change in gonadotropin gene *Lhb* and *Fshb* mRNA levels in the offspring at different ages from postnatal day 8 up to 13 months.

Introduction

Exposure to endocrine disrupting chemicals (EDCs) could cause alterations and dysfunctions in the body's various organs and systems including male and female reproductive disorders, obesity, type-2 diabetes, adrenal disorders, thyroid disorders, and growth hormone disruptions (Yilmaz et al. 2019). Although humans and animals are constantly exposed to EDCs throughout life, there are some critical windows during which the body goes through intensive tissue proliferation, differentiation, and maturation. The embryonic period is one of the critical windows as it's the period when the major organs are forming and developing, and the effects of embryonic EDC exposure could last long until adult life (Horzmann et al. 2021) and even pass on to the next generations (Zhou et al. 2017b; Brehm and Flaws 2019; Wen et al. 2020).

Phthalates are a type of EDCs that are widely used as plasticizers and solvents in daily products including plastics, personal care products, cosmetics, and building materials. The phthalate family is made up of a group of chemicals with different molecular weights and ten commonly used phthalates include lower molecular weight phthalates (ester side chain containing 1 to 4 carbons)- dimethyl phthalate (DMP), diethyl phthalate (DEP), and dibutyl phthalate (DBP), as well as higher molecular weight phthalates (ester side chain containing more than 4 carbons)- diisobutyl phthalate (DiBP), benzylbutyl phthalate (BzBP), dicyclohexyl phthalate (DCHP), di(2-ethylhexyl) phthalate (DEHP), di-n-octyl phthalate (DnOP), di-isononyl phthalate (DiNP), and di-isodecyl phthalate (DiDP) (Wang et al. 2019). Phthalates are commonly known as anti-androgens as they could disrupt androgen steroidogenesis (Borch et al. 2006). Besides, they have also been reported to be the ligands of various receptors associated with toxicants exposure including aryl hydrocarbon receptor (AhR) (Ernst et al. 2014; Ge et al. 2022) and peroxisome proliferator-activated receptors (PPAR) (Latini et al. 2008). Although numerous studies investigated impacts of different types of phthalates on multiple organs including male and female gonads, thyroid gland, adrenal gland, and the pituitary gland, most of them focused on the effects of single phthalates such as DEHP, DiNP and BzBP (Barakat et al. 2017; Forner-Piquer et al. 2019; Warner et al. 2019). However, studies analyzing phthalate metabolite components in human urine samples revealed that humans are exposed to mixture of different phthalates rather than a single type (Silva et al. 2007; Dong et al. 2017). It’s important to understand the effects of phthalate mixtures relevant to human daily exposure levels as it is more relevant to real life phthalate exposure in humans.

Recently, there has been an emergence of studies focusing on the impacts of exposure to environmentally relevant phthalate mixtures on different organs including ovaries and uterus (Warner et al. 2019; Li et al. 2020; Gill et al. 2021), prostate development (Scarano et al. 2019), reproductive aging (Brehm and Flaws 2021), as well as transgenerational effects (Brehm et al. 2020; Li et al. 2020). However, very few studies investigated the impacts of environmentally relevant phthalate mixtures on the pituitary gland, which is the master gland regulating multiple endocrine functions. Previous studies have reported effects of single phthalates such as DEHP on the pituitary, which include changes in gonadotropin mRNA levels (Pocar et al. 2012) and estrogen receptor (ER) expression levels (Pérez et al. 2020). Those findings from single phthalates studies led to the demand for understanding the impacts of environmentally relevant phthalate mixtures on the pituitary compared to single phthalates.

In this project we focused on the effects of embryonic exposure to environmentally relevant phthalate mixtures on the reproductive functions in the offspring in both sexes at the level of the pituitary, specifically the changes of gonadotropin gene- luteinizing hormone subunit beta (*Lhb*) and follicle stimulating hormone subunit beta (*Fshb*) expressions at different ages in the offspring in both sexes.

Materials and methods

Chemicals

The environmentally relevant phthalate mixture includes DEP (35.22 %), DEHP (21.03 %), DBP (14.91 %), DiBP (8.61 %), DiNP (15.10 %), and BzBP (5.13 %). The concentration of each phthalate was determined by measurement of phthalate metabolites in the urine samples of pregnant women in central Illinois (Pacyga et al. 2021). The phthalate mixture was mixed with tocopherol-stripped corn oil which was also used as vehicle control. The doses used for the study were 20 μg/kg/day, 200 μg/kg/day, and 200 mg/kg/day and the justification of the doses used in relevant to human daily exposure levels were described in previous paper published by our collaborators (Zhou et al. 2017a).

Animals and treatments

CD-1 mice were housed at the University of Illinois at Urbana-Champaign, Veterinary

Medicine Animal Facility in polysulfone cages under 12-h light-dark cycles with temperature maintained at 22 ± 1 °C. All the experimental procedures regarding animal uses were approved by The Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign.

Females (n = 54) were mated with untreated males and the presence of vaginal sperm plug marked the date of gestational day 0.5 (G0.5). On G10.5, pregnant dams were randomly assigned into four treatment groups including vehicle control, 20 µg/kg/day, 200 µg/kg/day, and 200 mg/kg/day phthalate mixture dosing groups (13-14 dams/treatment group). The treatment was given orally once a day at the same time from G10.5 till the dams gave birth. The exposure window was chosen based on the period of ovarian development in the mouse as described in previous published paper (Zhou et al. 2017a). Dosing was performed in the lab of Dr. Jodi Flaws by her lab members.

Pituitary collection

On postnatal days 8, 21, 90, 6-month and 13-month, at least one male and one female pup per litter were randomly chosen for tissue collection. To eliminate the effects estrogen fluctuation during estrous cycle, female offspring were collected during diestrus. Different organs and tissues including serum blood, ovaries, uterus, intestine, brain, and testes were collected at the same time by collaborators. Our lab collected the pituitary gland at each time point for analysis. The pituitary glands from each offspring were removed from the base of the skull and snap frozen on dry ice, then stored in -80°C freezer until ready for tissue preparation.

RNA isolation and cRNA preparation

RNA isolation from frozen pituitaries was performed by the standard Trizol (Invitrogen) extraction method. Total RNA was reverse-transcribed according to the manufacturer's instructions using the ProtoScript Strand cDNA Synthesis kit (New England Biolabs) as our lab has previously described (Moran et al. 2012).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Pituitaries of each sex from each treatment group (n = 5) were prepped for qRT-PCR analysis. Oligonucleotide primers for *Lhb* and *Fshb* (Life Technologies, primer sequence see Table 1) were used to amplify gene-specific transcripts by qPCR. The expression levels of genes of interest were normalized to *Ppia* mRNA levels. *Ppia* is a stable housekeeping gene for qRT-PCR normalization (Muñoz et al. 2021) that we confirmed had consistent expression levels across different treatments The data were analyzed using the standard comparative cycle threshold value method ($2^{-\Delta\Delta Ct}$) as previously described (Goldberg et al. 2011).

Table 4.1: PCR primers used in this study

Gene	Forward Sequence 5'-3'	Reverse sequence 3'-5'
Ppia	CAAATGCTGGACCAAACACAAACG	GTTCATGCCTTCTTTCACCTTCCC
Fshb	TGGTGTGCGGGCTACTGCTAC	ACAGCCAGGCAATCTTACGGTCTC
Lhb	CCCAGTCTGCATCACCTTCAC	GAGGCACAGGAGGCAAAGC

Statistical analysis

Male and female data were analyzed separately, and each pup from different litter was considered as one unit statistically. All data were presented as mean +/- SEM. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparisons test. P values less than 0.05 were considered significant. All analyses were performed using Graph Pad Prism 9.2.0.

Results

Gonadotropin mRNA levels were not changed in the offspring pituitary at different ages

To determine whether the prenatal exposure to an environmentally relevant phthalate mixture at different doses would affect the offspring's reproductive functions at the level of the pituitary, we examined the gonadotropin genes (*Lhb* and *Fshb*) mRNA expression levels in the pituitary at the age of P8 (neonatal period), P21 (pre-pubertal period), P90 (mature adults), and 13 months (aged, females only) in both male and female offspring. We found no significant difference between different phthalate mixture dosing group (20 μg, 200 μg, and 200 mg/kg/day) and control group's gonadotropin gene mRNA levels at different time points in neither male (Figure 4.1) or female (Figure 4.2) offspring.

Discussion

The absence of mRNA level differences between prenatal phthalate mixture exposed offspring and control group indicates the possibility that there's no substantial change caused by the phthalate mixture at the level of the pituitary. This result is interesting comparing to data reported from previous studies showing prenatal and lactational exposure to the single phthalate DEHP (50 μg and 5 mg/kg/day) in mice could cause gonadotropin gene mRNA levels to increase in both sexes at the age of P42 (Pocar et al. 2012). One explanation for the different outcome between different studies could be attributed to different exposure windows, as in the previous study the offspring were dosed with DEHP throughout whole embryonic and lactational period instead of only e10.5- e18.5. Another possibility could be that different types of phthalates in the mixture could have counteracting effects on each other in terms of gonadotropin mRNA expression and this would result in the unchanged *Lhb* and *Fshb* mRNA levels. For example, prenatal exposure to DBP (100 and 500 mg/kg/day) from e12.5 to e21.5 in rats was reported to decrease *Lhb* mRNA levels at the age of P3, 7, 14 and 21, and 100 mg/kg/day DBP dosage increased the

Lhb level at the age of P28 and P56 (Chen et al. 2017), however, DEHP was found to increase *Lhb* (Pocar et al. 2012).

Although we didn't see a change in gonadotropin mRNA levels, there could be changes in serum LH and FSH levels or LHβ and FSHβ protein level changes. Future studies should further investigate the serum level and protein level of the gonadotropins.

Figures

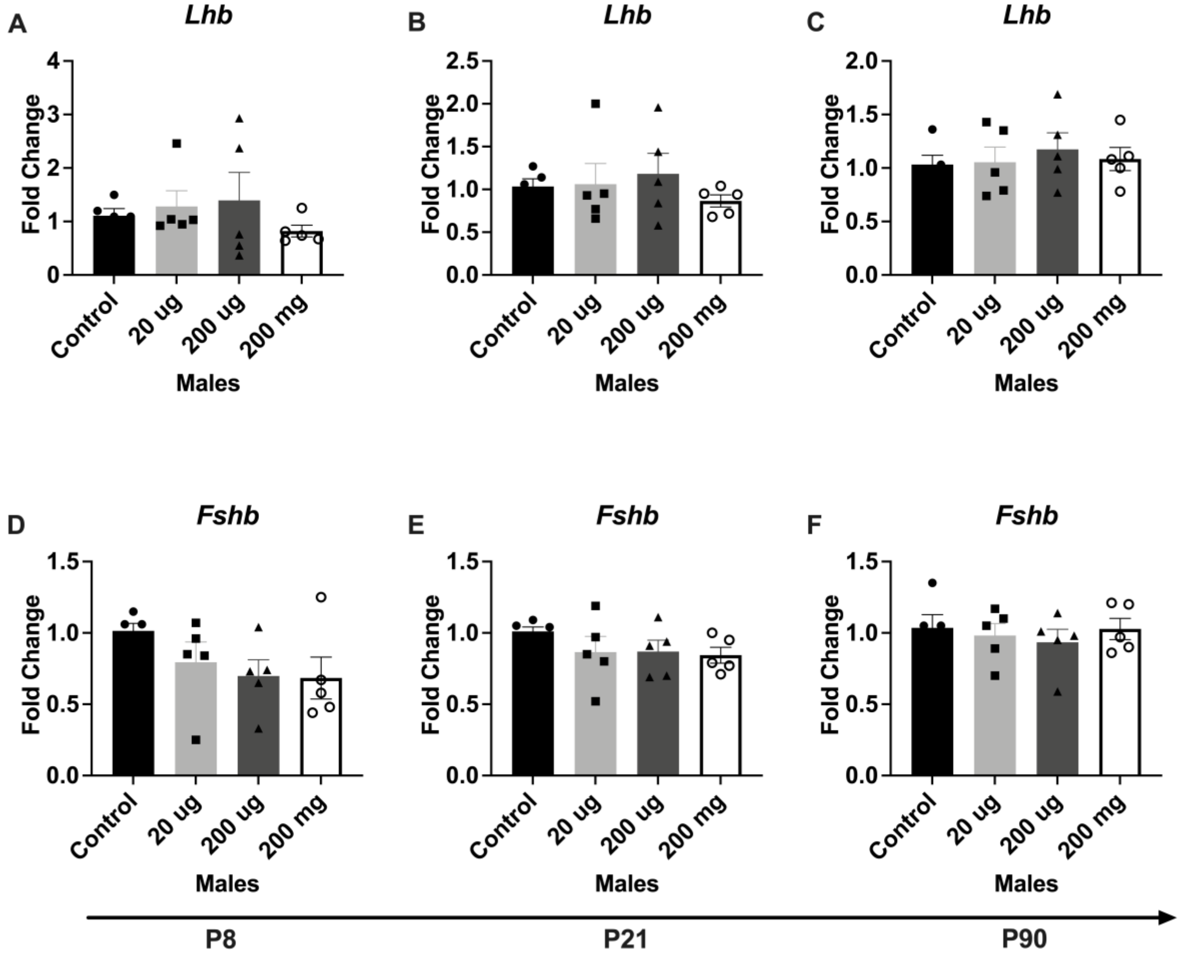

Figure 4.1 Pituitary gene expression levels of Lhb and Fshb mRNA in the male offspring in response to phthalate mixture exposure at different concentrations (vehicle control, 20 µg/kg/day, 200 µg/kg/day, and 200 mg/kg/day) at P8(A, D), P21(B, E), and P90 (C,F). The graph represents the mean +/− SEM (n = 5/treatment group). One-way ANOVA followed by Dunnett's multiple comparison's test found no significance in any condition.

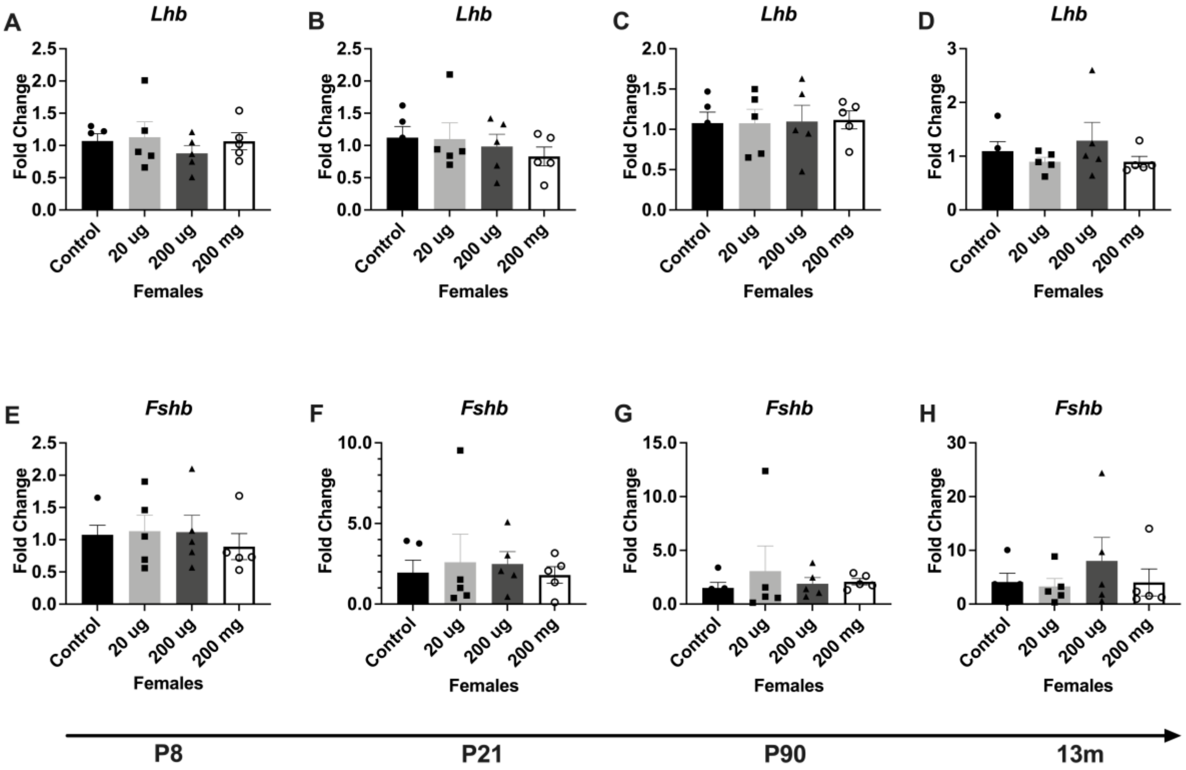

Figure 4.2 Pituitary gene expression levels of Lhb and Fshb mRNA in the female offspring in response to phthalate mixture exposure at different concentrations (vehicle control, 20 µg/kg/day, 200 µg/kg/day, and 200 mg/kg/day) at P8 (A,E), P21 (B, F), P90 (C, G), and 13-month (D, H). The graph represents the mean +/− SEM (n = 5/treatment group). One-way ANOVA followed by Dunnett's multiple comparison's test found no significance in any condition.

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CHAPTER 5: CONCLUSIONS AND DISCUSSION

As the master gland regulating the endocrine system, the pituitary mediates numerous physiological processes. Dysfunctions in the gland can cause diseases including hypopituitarism, a deficiency in pituitary hormone secretion that leads to disrupted endocrine functions in multiple systems. The causes of over 80% of hypopituitarism cases remain unknown, which indicates novel genes and mechanisms yet to be discovered can contribute to the disease. Pituitary development is a critical process, as any disruptions during the period could lead to abnormal pituitary formation and function, that eventually could be the cause of hypopituitarism or other pituitary diseases. The regulation of pituitary development comprises two parts: intrinsic signals within the developing pituitary, including sequential expression of transcription factors and involvement of multiple signaling pathways; and extrinsic signals from other endocrine organs as well as aberrant environmental signals, including exposure to endocrine disrupting chemicals. Both intrinsic and extrinsic signals are crucial for pituitary development and understanding of their mechanisms of action could help us to develop treatments for hypopituitarism. The studies described in Chapter 2 focused on intrinsic signals, specifically a novel marker for pituitary stem cells and its regulation and functions in the developing pituitary. Chapter 3 and Chapter 4 focused on extrinsic signals, specifically exposure to one or a mixture of phthalates and their impacts on gonadotroph functions during development.

GPHA2 is a novel marker for pituitary stem cells and is regulated by NOTCH signaling

Although NOTCH signaling is known to be crucial for pituitary development including stem cell proliferation, maintenance, and differentiation (Monahan et al. 2009; Nantie et al. 2014), the mechanisms by which NOTCH signaling exerts its functions in the pituitary remains unclear. In Chapter 2 we identified a novel stem cell marker, glycoprotein hormone subunit alpha 2 (GPHA2), that is regulated by NOTCH signaling. We identified expression of *Gpha2* in pituitary stem cells from multiple single-cell RNA sequencing (scRNA-seq) datasets and confirmed the expression of *Gpha2* in stem cells by RNAscope *in situ* hybridization (ISH) staining showing co-localization of *Gpha2* and the stem cell marker *Sox2* at multiple ages during development in mice. Notably, both scRNA-seq data and RNAscope ISH staining showed expression of *Gpha2* only in a subset of stem cells. scRNA-seq data showed different clusters of stem cells including proliferating (*Mki67*+) and non-proliferating stem cells, and the majority of *Gpha2* expressing cells were non-proliferative. The expression pattern of *Gpha2* is the same as *Notch2* according to scRNA-seq data. From RNAscope ISH staining, we observed expression of *Gpha2* in the intermediate lobe (IL) and anterior lobe (AL) stem cells, but interestingly, *Gpha2* expression in the AL is only present in the parenchyma stem cell niches, but not in the marginal cell layer (the "cleft"). The differences between these two types of stem cell niches have been reported in studies on rodents: stem cells in the cleft are considered primary niches that appear early in development and decrease in number in adulthood; parenchyma stem cells are considered secondary niches that originate from the primary niches and increase in number after birth (Gremeaux et al. 2012). However, more detailed functional differences between the two types of stem cells are still unclear. To further investigate the functions of GPHA2 in the pituitary, the characteristics of parenchyma stem cells should be considered when forming the hypothesis as the distinct expression pattern of *Gpha2* suggests its functions are related to parenchyma stem cells.

In addition, we reported *Gpha2* expression in the pituitary being regulated by *Notch2* because of reduction of *Gpha2* in *Notch2*-cKO pituitaries. However, our current data cannot show whether *Notch2* directly regulates *Gpha2* or acts through other mediators that eventually reduce the *Gpha2* expression. Future studies could investigate if there's direct regulation of *Gpha2* by *Notch2* by applying techniques such as chromatin immunoprecipitation sequencing (ChIP -seq) or CUT&RUN that look for possible binding of the NOTCH transcription regulator RBPJ in the regulatory region of *Gpha2* gene.

GPHA2 activates TSHR signaling in the pituitary

In terms of functions of GPHA2 in the pituitary, our study is the first to identify GPHA2 as a ligand for thyroid stimulating hormone receptor (TSHR). We showed induction of a downstream target of TSHR signaling- phospho-CREB (pCREB) protein in both neonatal and adult pituitaries and inhibition of pCREB induction by co-treatment with TSHR inhibitor in dissociated pituitary cells. Previous literature reported thyrostimulin, a heterodimer glycoprotein hormone composed of GPHA2 and glycoprotein hormone subunit beta 5 (GPHB5), functioned in a paracrine manner signaling through TSHR in the ovary (Sun et al. 2010). However, GPHA2 has also been reported to act as a monomer (Collin et al. 2021), which would parallel what we would predict how it would act in the pituitary. We think GPHA2 could be a part of the pituitary stem cell secretome that includes WNT signals that act in a paracrine manner on neighboring progenitor cells to regulate their proliferation (Russell et al. 2021). As mentioned in a previous section, Gpha2 is located in parenchymal stem cell niches where the stem cells could play a role in the cell turnover regulation required for postnatal anterior pituitary expansion (Yoshida et al. 2016). Taking these data together, we hypothesize GPHA2 act in a paracrine manner through TSHR signaling on neighboring cells to regulate their proliferation in the postnatal pituitary, which could be a mechanism by which NOTCH signaling functions to regulate postnatal gland maintenance. However, our current data haven't indicated the cell type where TSHR signaling is activated by GPHA2, and this could be a direction for future study. One way to identify the cell type is through immunohistochemistry (IHC) to co-stain pCREB with markers for different pituitary cell types and co-expression would indicate the induction of pCREB in certain cell type. However, given the basal expression of pCREB we observed from our staining, the cell type where pCREB is induced might be hard to identify. Another option is to apply RNAscpe ISH to identify co-expression of *Tshr* with marker genes for different cell types since the expression of *Tshr* would indicate the cell to be a target for GPHA2. In future studies, these techniques could both be applied to identify target cells of GPHA2 in the pituitary.

In addition to GPHA2's possible functions as a paracrine factor, recent studies revealed GPHA2 as a marker in quiescent stem cells in the cornea and determined that it functions to keep the undifferentiated state of the stem cells (Altshuler et al. 2021; Collin et al. 2021). These data provide other perspectives for understanding GPHA2's functions in the pituitary. Bromodeoxyuridine (BrdU) and phospho-histone H3 (pH3) staining could be used to identify cell division state and quiescence state of cells (Nantie et al. 2014), and they could be applied in future studies investigating GPHA2 in relation to stem cells' quiescence in both GPHA2-overexpressing of GPHA2-knockout conditions. For GPHA2's functions in regulating the undifferentiated state of stem cells, the stem cell colony formation assay could be applied to identify stem cells' state of differentiation, and this could be followed by knocking down of *Gpha2* (*Gpha2* siRNA) to see if the colony formation changes (Collin et al. 2021).

DEHP activates AhR signaling in developing pituitary and impacts gonadotroph functions

Our study investigated the endocrine disrupting chemical DEHP, and its primary metabolite MEHP, as extrinsic signals that could interfere with pituitary development and function. We reported DEHP's ability to interfere with androgen signals in males after exposure by inducing *Lhb* expression in the pituitary during the prenatal androgen surge period. Furthermore, our study is the first to report activation of aryl hydrocarbon receptor (AhR) signaling by MEHP directly in the pituitary by showing induction of AhR target genes *Cyp1a1* and *Cyp1b* in embryonic pituitary explant culture as well as induction of transcription through xenobiotic response element (XRE) in GHFT1 cells. Induction of *Cyp1b1* could be reversed by co-treatment with an AhR antagonist in explant culture. In addition, we observed reduction of both gonadotropin mRNAs by MEHP in pituitary cultures and the reduction was not ablated by co-treatment with AhR antagonist. Our findings of AhR activation and downregulating of gonadotropins indicate an association between AhR signaling and functioning of gonadotrophs, which can be supported by previous research reporting endogenous AhR signaling is required for normal functional of reproductive system

(Barnett et al. 2007). However, unresponsiveness of gonadotropin mRNAs to the AhR antagonist indicates the downregulation of gonadotropins is not directly associated with AhR activation in the pituitary, so the mechanism is unknown. Future studies could focus on investigating the role of AhR signaling the development and functions of gonadotrophs, which could start with identifying the AhR expressing cell types and their association with gonadotrophs.

Another interesting topic from our studies is that as target genes of AhR, *Cyp1a1* and *Cyp1b1*, had different responses to both DEHP/MEHP as well as AhR antagonist treatment. The non-monotonic dose response of *Cyp1a1* to MEHP in pituitary culture suggests an explanation for unchanged *Cyp1a1 in vivo*: the dosage of DEHP the pituitary gets exposed to may be critical for gene expression. This hypothesis leads to the need for understanding the mechanisms of EDCs' low-dose responses or nonmonotonic dose responses (Vandenberg et al. 2012). Although the pituitary is a well-vascularized organ, the exact dosage of EDCs that came in direct contact with the pituitary versus the dosage in blood or urine samples, which is often the parameter for setting the dosage of *in vitro* experiments, is unknown. Research questions focusing on the metabolism of the EDCs in human and animal models and specifically local concentration of EDCs near the pituitary need to be answered.

We also observed sex differences in response to developmental DEHP exposure, which is a common topic of EDC exposure. Sex differences are important factors known to cause different responses to EDC exposure between males and females in the hypothalamic-pituitary axis, specifically in reproductive-related functions which have the most prominent distinctions between sexes (Patisaul 2021). There are not only sexually dimorphic regions in the hypothalamus that could secret different signals to the pituitary gland in response to EDC exposure, but also sex differences in the developing pituitary gland transcriptome (Bjelobaba et al. 2015; Eckstrum et al. 2016; Qiao et al. 2016; Hou et al. 2017). These differences in pituitary gene expression could be the starting points for understanding how developmental exposure to EDCs could directly impact the pituitary differently between sexes.

Overall conclusions

In summary, our studies provided novel findings for understanding pituitary development and proper functioning from both intrinsic and extrinsic perspectives. We identified GPHA2 as a potential NOTCH target and provided data showing its activation of the TSHR signaling pathway and its possible functions. We also reported the activation of AhR in pituitary by DEHP, which could be a potential signaling pathway through which extrinsic signals regulate gonadotroph development. Therefore, our studies will help in understanding the intricate regulation of pituitary development and the mechanisms of pituitary diseases including hypopituitarism.

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