The regulation of the luteinizing hormone (LH) and the follicle stimulating hormone (FSH) by glucocorticoids and progestins

Carole-Keza Capitaine

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Supervisor: Prof. Janet P. Hapgood

Co-supervisor: Dr. Alexis Bick

Abstract

Estradiol (E2) plays a crucial role in female reproduction and in the defense against HIV-1 in the female genital tract (FGT). Medroxyprogesterone acetate (MPA) intramuscular (DMPA-IM) (Depo-Provera) and norethisterone (NET) enanthate (NET-EN) are injectable progestin only contraceptives. DMPA-IM is commonly used in sub-Saharan Africa, while NET-EN is commonly used in South Africa. Controversial observational studies have demonstrated a heightened susceptibility to HIV-1 acquisition associated with the use of DMPA-IM but not NET-EN. The recent Women's Health, Injectable Contraception and HIV (WHICH) clinical trial compared E2 levels in equal numbers of women randomized to DMPA-IM (n=262) and NET-EN (n=259) found that both DMPA-IM and NET-EN use caused hypoestrogenism. Considering the widespread use of DMPA-IM and NET-EN among South African women, who are disproportionately affected by HIV-1, it is important to explore the mechanisms through which these contraceptives induce hypoestrogenism. These could potentially involve luteinizing hormone (LH), follicle-stimulating hormone (FSH), and gonadotropin-releasing hormone (GnRH), all hormones involved in the hypothalamic-pituitary-ovarian (HPO) axis and key regulators of E2 synthesis and release.

This thesis analyzed the serum concentrations of E2, LH, FSH, and GnRH in women randomized to DMPA-IM (n = 100) or NET-EN (n = 93) in a subpopulation of the WHICH cohort. This thesis also involved a translational approach, comparing clinical data with mechanistic data from an in vitro model. Towards understanding the mechanisms whereby MPA and NET may regulate gonadotropin levels, it was hypothesized that these progestins exert direct actions on pituitary gonadotropes. It was further hypothesized that while both progestins are progestogenic and could act via the progesterone receptor (PR), MPA would most likely also exert glucocorticoid-like actions via the glucocorticoid receptor (GR), unlike NET. These hypotheses were tested in the LβT2 mature mouse pituitary gonadotrope cell line. This in vitro model allowed the effects of MPA and NET on the gonadotropins at a transcriptional, post-transcriptional and secretion level to be tested. This study also investigated the effects of MPA, NET, dexamethasone (DEX, a GR agonist) and progesterone (P4, a PR agonist), in the absence and presence of GnRH, on LH and FSH regulation in the LβT2 cells.

Clinical results showed that E2 levels were similarly suppressed to postmenopausal levels by both contraceptives in the subpopulation of women. No effects were detected for either contraceptive on GnRH levels. LH levels decreased in both contraceptive groups, whereas FSH levels decreased in the NET-EN group and increased in the DMPA-IM group. The results in LβT2 cells showed that MPA and DEX both suppressed the GnRH-induced promoter-reporter activity of the beta subunit of LH (LHβ), with a non-significant decrease in GnRH-induced LH protein secretion. MPA and DEX increased the promoter-reporter activity and mRNA level of the beta subunit of FSH (FSHβ), while NET and P4 had no detectable change on FSH beta expression. Additionally, both MPA and DEX enhanced basal and GnRH-induced FSHβ promoter activity and mRNA levels. Taken together with GR antagonist experiments, the effects of MPA and DEX on FSHβ gene expression are likely mediated by the GR. Attempts to measure secreted FSH protein were unsuccessful.

In summary, the combined clinical and in vitro data suggest that the hypoestrogenic effect caused by DMPA-IM and NET-EN in women is likely through direct actions of MPA and NET on pituitary gonadotropes to decrease LH levels. The decrease in LH levels in DMPA-IM users occurs most likely both at the level of gene transcription, as well as at the level of protein secretion, while this does not appear to be the case for NET-EN users. The increase in FSH levels in DMPA-IM users is likely due to direct effects by MPA on pituitary gonadotropes to increase FSHβ transcription and mRNA levels. This increase in FSH levels is likely due to the GR-mediated transcriptional regulation of FSHβ by MPA and not NET. The in vitro data do not explain the decrease in FSH levels detected in NET-EN users. However, there may be other unexplored mechanisms used by MPA and NET at the level of FSH secretion. The data also do not exclude that DMPA-IM and NET-EN may have other mechanisms that act at the ovarian level or in the hypothalamus or above the hypothalamus, to affect GnRH pulsatility. This study gives insight into previously unknown mechanisms involved in the regulation of gonadotropins by MPA and NET. This research also enhances our understanding of the potential mechanisms behind the hypoestrogenic effects caused by progestin-only injectable contraceptives.

Plagiarism declaration

I know the meaning of plagiarism and declare that all of the work in the dissertation (or thesis), save for that which is properly acknowledged, is my own.

Signed by candidate

Carole-Keza Capitaine

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"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale." One never notices what has been done; one can only see what remains to be done."- Marie Curie

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List of Abbreviations

α-GSU: alpha- glycoprotein subunit

ACTH: adrenocorticotropic hormone

ACT-RII: type II activin receptor

AR: androgen receptor

CgA: chromogranin A

Cga: chorionic gonadotropin alpha polypeptide

ChIP: chromatin immunoprecipitation

COC: combined oral contraceptives

CRF: corticotropin releasing factor

CYP-19: aromatase cytochrome P450 family 19

DEX: dexamethasone

DMPA-IM: depo medroxyprogesterone acetate Intramuscular

DHT: dihydrotestosterone

ECHO: Evidence for Contraceptive Options and HIV outcomes

EE: ethinyl estradiol

ELISA: enzyme-linked immunosorbent assay

FGT: female genital tract

FSH: follicle-stimulating hormone

FSH-R: follicle-stimulating hormone receptor

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GnRH: gonadotropin-releasing hormone

GnRH-R: gonadotropin-releasing hormone receptor

GR: glucocorticoid receptor

HIV-1: human immunodeficiency virus type 1

HPA: hypothalamic-pituitary-adrenal

HPO: hypothalamic-pituitary-ovarian

HRE: hormone responsive element

hCG: human chorionic gonadotropin

IGF-1: insulin growth factor-1

IUD: intra uterine device

LβT2: luteinizing hormone β subunit-expressing T2 mouse pituitary cells

LH: luteinizing hormone

LH-R: luteinizing hormone receptor

LNG: levonorgestrel

MEC 1: Medical Eligibility Category 1

MPA: medroxyprogesterone acetate

NET-EN: northisterone enanthate

P4: progesterone

PKA: protein kinase A

RFRP3: RFamide-related peptide-3

RT-qPCR: reverse transcription quantitative polymerase chain reaction

Sg II: secretogranin II

SR: steroid receptor

SSA: sub-Saharan Africa

T: testosterone

TBP: TATA-box binding protein

VPN: vasopressin

UNAIDS: Joint United Nations Programme on HIV/AIDS

WHICH: Women's Health, Injectable Contraception and HIV

WHO: World Health Organization

Chapter 1: Literature review

1.1 Injectable contraceptives, HIV-1 and hypoestrogenism

One of the most popular hormonal contraceptives used in sub-Saharan Africa (SSA) is depo medroxyprogesterone acetate intramuscular (DMPA-IM) (Ayuk *et al.*, 2022). Additionally, norethisterone enanthate (NET-EN) is another widely used contraceptive in South Africa (Ayuk *et al.*, 2022). Both hormonal contraceptives are injectable and contain only synthetic progestins, unlike other combined hormonal contraceptives that contain both progestins and an estrogen, typically ethinyl estradiol (EE). The synthetic progestins for DMPA-IM and NET-EN are medroxyprogesterone acetate (MPA) and norethisterone (NET), respectively. DMPA-IM is administered every 3 months at a dose of 150 mg, while NET-EN is administered every 2 months at a dose of 200 mg (Goebelsmann *et al.*, 1979; Jeppsson *et al.*, 1982). These contraceptives have gained popularity owing to their effectiveness in the prevention of pregnancy (Ayuk *et al.*, 2022). Additionally, DMPA-IM is widely accessible in SSA, while NET-EN is widely available in South Africa, and more specifically, these contraceptives are easily accessible in clinics located in remote locations (Ayuk *et al.*, 2022). Given the prevalent stigma associated with contraceptive use in these areas, this option enables women to discreetly manage family planning. The advantage lies in the fact that a single dose can provide protection for several months, eliminating the need for daily medication. Furthermore, these contraceptives are deemed safe (Ayuk *et al.*, 2022).

However, both clinical observational studies and biological data suggest an increased risk of HIV-1 acquisition for women using DMPA-IM compared to women using NET-EN or combined oral contraceptives (COC) (Morrison *et al.*, 2015; Polis *et al.*, 2016; Hapgood, Kaushic and Hel, 2018). This issue looms large, particularly in light of the prevailing HIV-1 infection rates in SSA. Notably, the prevalence of HIV-1 infections in SSA exhibits a significant gender disparity, with women being disproportionately affected (UNAIDS, 2022). Presently, 63% of all new infections in SSA occur in women (UNAIDS, 2022). This statistic is concerning, given that this is the same demographic of women who are also injectable contraceptive users in SSA (Ayuk *et al.*, 2022). From 2015 to 2017, the Evidence for Contraceptive Options and HIV Outcomes (ECHO) trial was conducted to compare HIV-1 incidence in women using DMPA-IM, levonorgestrel (LNG)

implant and the copper intrauterine device (IUD) (Ahmed *et al.*, 2019). The ECHO trial was designed to only detect a 50% or more increase in HIV-1 risk between the three contraceptive arms (Ahmed *et al.*, 2019). However, due to the lack of a no contraceptive arm, the trial was not able to measure absolute HIV-1 risk (Ahmed *et al.*, 2019; Hapgood, 2020). DMPA-IM was found to increase HIV-1 risk by 36% compared to the LNG implant and copper IUD (Ahmed *et al.*, 2019; Hapgood, 2020). However, due to the design of the trial, significant differences in HIV-1 acquisition risk between methods lower than 50% may not have been detected (Ahmed *et al.*, 2019; Hapgood, 2020). Furthermore, the threshold chosen to detect HIV-1 risk is controversial (Hapgood, 2020), given that other meta-analyses found DMPA-IM to increase HIV-1 risk by 40-50% compared to no hormonal contraception (mostly no contraception or infrequent condom use) (Morrison *et al.*, 2015; Polis *et al.*, 2016; Hapgood, Kaushic and Hel, 2018). Additionally, in vitro evidence shows that MPA can have immunosuppressive effects, thereby potentially increasing the risk of HIV-1 infection (Huijbregts *et al.*, 2013; Govender *et al.*, 2014; Hapgood, Kaushic and Hel, 2018; Maritz *et al.*, 2018). In the case of NET-EN, limited observational studies have indicated that there is no increased risk of HIV-1 infection with NET-EN compared to DMPA-IM or no hormonal contraceptives, while DMPA-IM increases HIV-1 risk by 32-40% compared to NET-EN (Lawrie *et al.*, 1998; Morrison *et al.*, 2015; Polis *et al.*, 2016).

There are multiple mechanisms associated with DMPA-IM that potentially increase HIV-1 acquisition risk. One of these mechanisms is hypoestrogenism (Hickey, Marino and Tachedjian, 2016; Hapgood, Kaushic and Hel, 2018). The female sex steroid estradiol (E2) has many functions in women. Primarily, E2 is vital for the reproductive functions of maintaining the menstrual cycle and inducing ovulation (Reed and Carr, 2000). Additionally, E2 provides protection against viral infections in the female genital tract (FGT) (Marx *et al.*, 1996; Smith, Baskin and Marx, 2000; Hapgood, Kaushic and Hel, 2018). E2 has the capacity to increase chemokine production and the secretion of anti-viral peptides (Smith, Baskin and Marx, 2000; Hapgood, Kaushic and Hel, 2018). Furthermore, E2 enhances the thickness of the vaginal epithelial mucosa, thus creating a barrier that prevents viruses from reaching cells beneath the epithelial lining (Cotreau *et al.*, 2007; Hummelen *et al.*, 2011). Lastly, E2 supports the growth of microbial lactobacilli species, which secrete lactic acid that breaks down viral membranes (Molander *et al.*, 1990; Hapgood, Kaushic and Hel, 2018). Therefore, reduced E2

levels in injectable contraceptive users would affect both reproductive functions and susceptibility to infections.

Previous studies have shown pre-menopausal women on DMPA-IM have E2 levels (69.4-374 pmol/L) in a range that overlaps E2 levels found in the early follicular levels (172.5-234.9 pmol/L) and comparable to those in post-menopausal women (7.3-158 pmol/L) (**Table 1.1**). The few studies that have investigated endogenous E2 levels in women on NET-EN found that NET suppressed E2 to an early follicular level (132.9-734.2 pmol/L) (Goebelsmann *et al.*, 1979; Saleh *et al.*, 1983). However, some studies show that E2 is suppressed more so in women on NET-EN compared to women not on hormonal contraceptives (Lawrie *et al.*, 1998; Dabee *et al.*, 2019). However this E2 suppression by NET-EN is not to the same degree as E2 suppression reported in DMPA-IM users (Hickey, Marino and Tachedjian, 2016). E2 levels in various studies in DMPA-IM and NET-EN users are shown in **Table 1.1** (Clark *et al.*, 2001; Hickey, Marino and Tachedjian, 2016). A detailed account of E2 concentrations for each reference can be found in **Addendum A Tables A1-A4**. Currently, only one study has compared the relative E2 levels in DMPA-IM users and NET-EN users (Dabee *et al.*, 2019). The most robust data to date from the Women's Health, Injectable Contraception and HIV (WHICH) clinical trial, measured and compared E2 levels in equal numbers of women randomized to DMPA-IM (n=262) and NET-EN (n=259) found that in both contraceptive groups, endogenous E2 was suppressed to concentrations that were comparable to E2 levels in post-menopausal women (Singata-Madliki *et al.,* 2024). DMPA-IM users had a median concentration of 76.5 pmol/L and NET-EN users had a median concentration of 69.8 pmol/L (Singata-Madliki *et al.*, 2024) (**Table 1.1**), showing that both DMPA-IM and NET-EN decrease E2 levels to similar concentrations and cause hypoestrogenism in pre-menopausal women.

Collectively, E2 not only serves as a crucial hormone necessary for the proper function of the reproductive system but also plays a major role in establishing a protective barrier against viral infections in the FGT. Therefore, the suppression of E2 by injectable progestin contraceptives is likely to decrease these protective functions. Considering that HIV-1 infection is highly prevalent amongst young women of reproductive age (UNAIDS, 2022), i.e., the demographic using these contraceptives, understanding the mechanisms responsible for hypoestrogenism induced by these contraceptives is important. This is because alternative contraceptive

choices or strategies could be introduced in order to mitigate the hypoestrogenic effect of these contraceptives, especially for high- risk populations.

Regulation of E2 levels is primarily orchestrated by the hypothalamic-pituitary-ovarian (HPO) axis. In order to comprehend how DMPA-IM and NET-EN suppress E2 levels, it is essential to understand the functioning of this axis, as the contraceptives may modulate E2 levels by influencing hormonal signals at one or more levels within the HPO axis.

Table 1.1: E2 in pre-menopausal women, post-menopausal women, and DMPA-IM and NET-EN users

Pre-menopausal		
E2 (pmol/L)	**Age and number of women**	**Reference**
275.3 -381.3 (phase unknown)[2]	18-46; n= 12-1690	Randolph *et al.*, 2004; Kaaks *et al.*, 2005; Hutchens *et al.*, 2016
172.5-234.9 (early follicular phase)[2] 249.6-385.5 (mid-follicular phase)[2] 444.6-881 (late follicular phase)[2]	20-46; n=5-23	Soules *et al.*, 1984; Welt *et al.*, 1999; Hutchens *et al.*, 2016
403.8-488.2 (early luteal phase)[2] 521.3-587.4 (mid-luteal phase)[2] 374.4-403.8 (late luteal phase)[2]	20-46; n=5-23	Soules *et al.*, 1984; Welt *et al.*, 1999
Post-menopausal		
E2 (pmol/L)	**Age and number of women**	**Reference**
7.3-158[2]	40-65; n=30-1015	Ausmanas *et al.*, 2007; Kling *et al.*, 2019; Grub *et al.*, 2021; Kawakita *et al.*, 2023
DMPA-IM		
E2 (pmol/L)	**Age and number of women**	**Reference**
79 (56-108)[1]	18-40; n=99	Singata-Madliki *et al.*, 2024

69.4 – 374[2]	15-43; n=1- 121	Briggs and Briggs, 1972; Mishell *et al.*, 1972; Jeppsson and Johansson, 1976; Jeppsson *et al.*, 1977, 1982; Mishell, 1996; Miller *et al.*, 2000; Clark *et al.*, 2001; Schaffir, Isley and Woodward, 2010; Hickey, Marino and Tachedjian, 2016; Siregar, Rita and Yusrawati, 2019
NET-EN		
E2 (pmol/L)	**Age and number of women**	**Reference**
59.85 (69.5-87.23)[1]	18-40; n=94	Singata-Madliki *et al.*, 2024
132.9-734.2[2]	20-40; n=9-73	Toppozada, Parmar and Fotherby, 1978; Fotherby *et al.*, 1980; Saleh *et al.*, 1983; Lawrie *et al.*, 1998

*[1]- Median (IQR)

[2]- Detailed concentration for each reference can be found in **Addendum A Table 1-4**

1.2 The hypothalamus-pituitary-ovarian (HPO) axis and the menstrual cycle

The HPO axis serves as the central regulatory network responsible for maintaining homeostasis of the female reproductive system in mammals (Acevedo-Rodriguez *et al.,* 2018). The HPO axis is comprised of three interconnected components: the hypothalamus, anterior pituitary gland, and ovaries. At the top of the axis, gonadotropin-releasing hormone (GnRH) is produced by the medial preoptic area and the arcuate nucleus in the hypothalamus (Casteel and Singh, 2022). GnRH is then secreted, in a pulsatile manner, through the hypophyseal portal vein. Subsequently, GnRH binds to its receptor, GnRH receptor (GnRH-R), in the gonadotroph cells of the anterior pituitary gland (Atwood and Vadakkadath Meethal, 2016). GnRH regulates the expression of the α and β subunits of the LH and FSH hormones, influencing their secretion (Burger *et al.*, 2004a). LH and FSH are released into the bloodstream, followed by binding to their respective receptors in the ovarian follicles (Raju *et al.*, 2013; Atwood and Vadakkadath Meethal, 2016). These hormones play critical roles in controlling follicular development and steroidogenesis and stimulating E2 and (to a lesser degree) P4 production during follicular development. Following ovulation, the follicle luteinizes to form the corpus luteum, which is

responsible for increased progesterone (P4) synthesis (Raju *et al.*, 2013; Atwood and Vadakkadath Meethal, 2016).

The HPO axis is regulated by positive and negative feedback mechanisms (**Figure 1.1**). The feedback mechanisms can act on either the hypothalamic level (GnRH), on a pituitary level (LH and FSH), on both levels, or even at levels of the brain above the hypothalamus (Whirledge and Cidlowski, 2010). These feedback mechanisms are typically dependent on the concentrations of the ovarian hormones (Joseph and Whirledge, 2017). A low concentration of E2 initiates positive feedback on both the hypothalamus and pituitary to increase GnRH, LH, and FSH, while the positive feedback caused by low P4 concentrations acts primarily at the pituitary to increase LH and FSH. Conversely, high concentrations of E2 create a negative feedback on both the hypothalamus and pituitary, in order to inhibit the expression and secretion of GnRH, LH and/or FSH (Joseph and Whirledge, 2017). Similarly, high concentrations of P4 exert a negative feedback on the hypothalamus to decrease GnRH and ultimately decrease the gonadotropins (Joseph and Whirledge, 2017). However, it is not only concentration that modulates the type of feedback mechanism exerted on GnRH and the gonadotropins, LH and FSH. The timing of when the ovarian hormones E2 and P4 are synthesized and released in relation to the particular phase of the menstrual cycle, also contributes to the type of feedback mechanism that is exerted on the hypothalamic and pituitary hormones. Thus, the type of feedback mechanism on the hypothalamic and pituitary hormones may depend on either the concentration of the downstream ovarian hormones, the phase of the menstrual cycle, or both of these factors (Joseph and Whirledge, 2017). It is also important to note that the evidence behind these feedback mechanisms in the HPO axis was largely studied in non-human mammals and not in women (Christensen *et al.*, 2012; Dagklis *et al.*, 2015; Joseph and Whirledge, 2017).

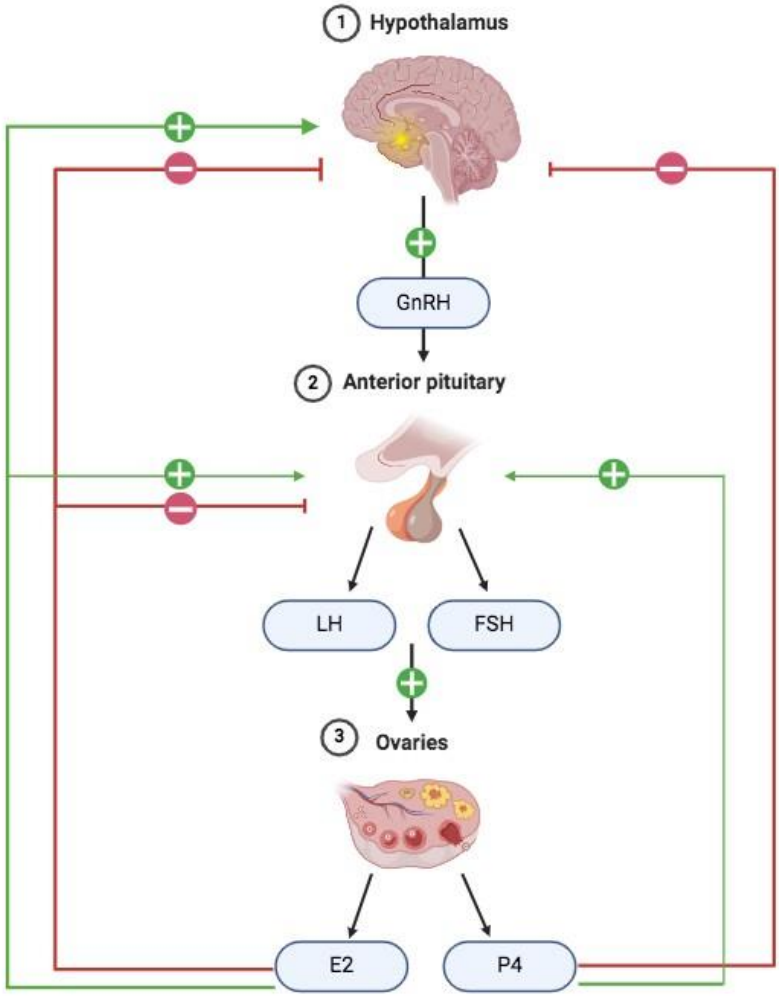


Figure 1.1: Regulation of the female mammalian HPO axis. *GnRH is produced and secreted in pulses from the hypothalamus to the anterior pituitary (Casteel and Singh, 2022). GnRH binds to the GnRH receptor in the gonadotroph cells of the anterior pituitary. GnRH stimulates and regulates the transcriptional, post-transcriptional and protein secretion of the glycoprotein hormones, LH and FSH (Burger et al., 2004a). Once secreted from the gonadotroph cells, LH and FSH travel via the bloodstream to the ovaries. LH and FSH will bind to their respective receptors in the ovary (Raju et al., 2013). Binding of LH to its receptor stimulates the production of P4 and testosterone (T) via steroidogenic enzymes, while FSH binding to its receptor stimulates the expression of the steroidogenic enzyme needed to convert T to E2 (Raju et al., 2013). Low concentrations of E2 stimulate a positive feedback mechanism at the hypothalamus and pituitary to increase the frequency of the GnRH pulse and the secretion of LH and FSH. Additionally, low P4 concentrations increase LH and FSH secretion by exerting a positive feedback mechanism at the pituitary (Hutchens et al., 2016). As the concentration of E2 increases, E2 induces negative feedback on the expression and secretion of FSH (Whirledge and Cidlowski, 2017). Conversely, E2 stimulates a positive feedback on the hypothalamus, which leads to an increase in GnRH pulse frequency and subsequently an LH surge (Whirledge and Cidlowski, 2017). Subsequently, the concentration of P4 increases and*

this creates a negative feedback on the hypothalamus and thus inhibits LH secretion (Soules et al., 1984; Nippolt et al., 1987; Messinisi, 2006). Created with BioRender.com.

The HPO axis orchestrates the cyclical production of both the pituitary and ovarian hormones (Davis and Hackney, 2017). This regulation by the HPO axis manages the hormonal fluctuations that are needed to maintain the structure and function of the menstrual cycle. The menstrual cycle consists of two distinct phases: the follicular phase and the luteal phase (Reed and Carr, 2000; Hawkins and Matzuk, 2008). Ovulation occurs at the end of the follicular phase before the luteal phase (Reed and Carr, 2000). During each of these phases, the HPO axis ensures regulation of the hormonal fluctuations in LH, FSH, E2, and P4 (Reed and Carr, 2000; Hawkins and Matzuk, 2008). In the follicular phase, the granulosa and theca cellular layers of the ovarian follicles develop and mature under the influence of LH and FSH (Reed and Carr, 2000; Hawkins and Matzuk, 2008). Subsequently, the development of these follicles leads to the production of E2. The subsequent rise of E2 concentrations in the mid-follicular phase signals positive feedback to the hypothalamus and pituitary. This results in an LH surge at the end of the follicular phase (Reed and Carr, 2000; Hawkins and Matzuk, 2008). This LH surge will cause the matured follicle to release an oocyte, or in other words, ovulation (Reed and Carr, 2000; Hawkins and Matzuk, 2008). During the luteal phase, under the stimulation of LH, the follicle will luteinize. This occurs when the granulosa cellular layer of the follicle enlarges and accumulates lutein and merges with the theca cellular layer, to form the corpus luteum (Reed and Carr, 2000). The corpus luteum subsequently produces increased P4 and E2. P4 and E2 exert a negative feedback at the hypothalamus and pituitary to decrease FSH and LH levels, in order to prevent the development of another follicle (Reed and Carr, 2000; Hawkins and Matzuk, 2008). Additionally, increased P4 levels result in the proliferation of capillaries, which leads to the vascularization of the endometrial lining (Cable and Grinder, 2023). This prepares the possible zygote for implantation (Reed and Carr, 2000). In cases of unfertilized ova, the corpus luteum undergoes degeneration, which leads to decreased P4 levels (Reed and Carr, 2000; Hawkins and Matzuk, 2008). The decreased P4 levels result in tissue ischemia in the endometrium. The remaining endometrial cells produce prostaglandins that promote the shedding of the endometrium lining (Reed and Carr, 2000; Hawkins and

Matzuk, 2008). Thus, menstruation or menses commences. At the end of menses, FSH levels increase, resulting in the start of a new cycle. This is represented in **Figure 1.2**. This intricate hormonal interplay shows that the production of E2 is regulated by multiple factors.

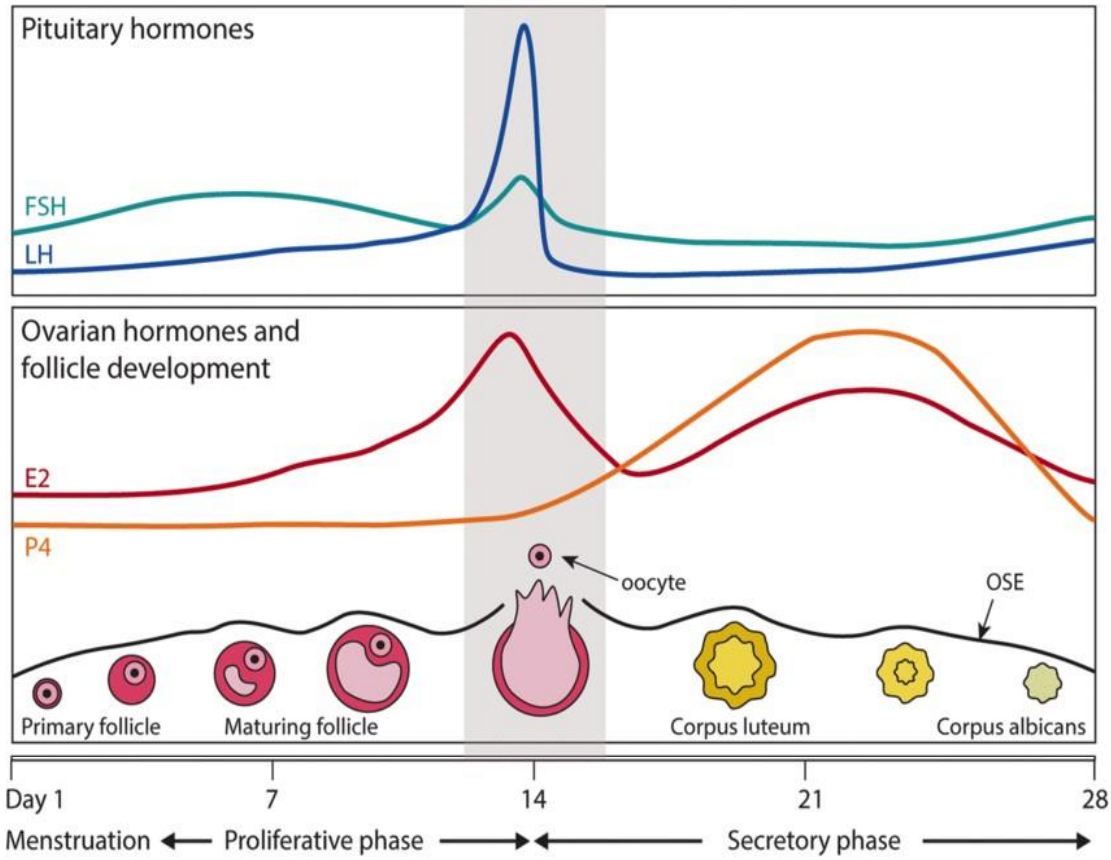


Figure 1.2: Schematic of the hormonal fluctuations and development of the follicle and endometrial lining across the menstrual cycle. *The menstrual cycle begins with the follicular phase (proliferative), where follicles mature under the stimulation of LH and FSH. As the follicle matures, E2 is synthesized and inhibits FSH to prevent further maturation of multiple follicles. Subsequently, an E2 surge occurs, which leads to the LH surge. The LH surge causes the dominant follicle to release the oocyte from the ovaries. Thus, ovulation occurs. The remaining luteinized follicle forms the corpus luteum during the luteinization phase (secretory phase). The corpus luteum secretes P4 and E2. P4 secretion inhibits LH and FSH secretion and thickens the endometrial lining. If an embryo is not formed, human chorionic gonadotropin (hCG) is not secreted. The development of the corpus luteum is dependent on hCG stimulation. Thus, the corpus luteum degenerates, and P4 secretion is inhibited. Therefore, the endometrial lining sheds, and menstruation occurs. Due to low levels of P4, LH and FSH are secreted, and a new cycle begins (Chumduri and Turco, 2021).*

A prevalent theme identified in the literature about the regulation and function of the HPO axis hormones is that the majority of evidence stems from animal studies. Moreover, there is insufficient evidence in women on the mechanisms that govern the regulation of these HPO axis hormones and their functions, which will be addressed in the subsequent sections.

1.3 Structure and function of LH and FSH

LH and FSH are produced in the gonadotroph cells of the anterior pituitary gland (Thackray, Mellon and Coss, 2010). These gonadotrophs can either produce only LH, only FSH or both hormones (Thackray, Mellon and Coss, 2010). Both hormones are glycoproteins, each consisting of two subunits: a commonly shared α subunit and different β subunits (Fan and Hendrickson, 2005; Cahoreau, Klett and Combarnous, 2015). The α subunit is highly conserved in the glycoprotein hormone family in multiple species (Fan and Hendrickson, 2005; Cahoreau, Klett and Combarnous, 2015). The α subunit mainly gives structure to both LH and FSH, but also has a high affinity for the respective glycoprotein hormone receptors (Fan and Hendrickson, 2005; Cahoreau, Klett and Combarnous, 2015). The β subunits are responsible for the biological specificity of LH and FSH. This subunit has high specificity for either the LH or FSH receptors (LH-R and FSH-R) (Fan and Hendrickson, 2005; Cahoreau, Klett and Combarnous, 2015). The chorionic gonadotrophin subunit alpha (Cga) or alpha glycoprotein subunit (α-GSU) gene expresses the α subunit, shared by the LH and FSH proteins. LHβ and FSHβ express the respective β subunit for the LH and FSH proteins (Thackray, Mellon and Coss, 2010) (**Figure 1.3**).

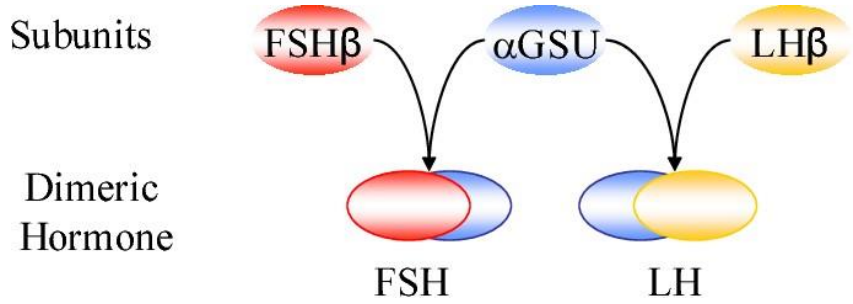


Figure 1.3: A schematic of the different subunits of LH and FSH. *LH and FSH share the α subunit and have different β subunits. The α subunit is expressed by the α-GSU or Cga gene, while the β subunits for FSH and LH are expressed by FSHβ and LHβ genes, respectively (Safwat, 2006).*

LH and FSH are key regulators of the general functioning of the ovaries by regulating steroidogenesis and folliculogenesis (Raju *et al.*, 2013). Folliculogenesis is linked to steroidogenesis. Both LH and FSH promote the development and maturation of ovarian follicles as part of maintaining the menstrual cycle. Through this development, the follicles will produce E2 and estrone (E1) (Raju *et al.*, 2013; Gervasio *et al.*, 2014).

1.3.1 Role of LH and FSH in E2 production

E2 synthesis throughout the menstrual cycle is tightly regulated by LH and FSH (Reed and Carr, 2000; Devoto *et al.*, 2002; Raju *et al.*, 2013). While FSH plays a dominant role in E2 synthesis during the follicular phase, LH plays a more dominant role in E2 synthesis during the luteal phase (Reed and Carr, 2000; Devoto *et al.*, 2002; Raju *et al.*, 2013).

1.3.1.1 E2 synthesis during the follicular phase

As mentioned above, folliculogenesis is intricately linked to steroidogenesis, a process directly regulated by LH and FSH (Raju *et al.*, 2013). Ovarian follicles consist of two distinct cellular layers: the granulosa and theca cells (**Figure 1.4**) (Miller and Auchus, 2011; Raju *et al.*, 2013). In the early follicular phase, FSH binds to the FSH receptor (FSH-R) in the granulosa cells and together with insulin growth factor 2 (IGF-2), promotes follicular growth (Reed and Carr, 2000; Raju *et al.*, 2013). This follicular growth leads to the proliferation of both granulosa and theca

cells (Reed and Carr, 2000; Miller and Auchus, 2011). The binding of FSH to FSH-R activates the cAMP-mediated protein-kinase A (PKA) pathway (Millier, Whitelaw and Smyth, 1994; Miller *et al.,* 2011), which in turn activates various transcription factors necessary for the expression of the key steroidogenic enzyme, aromatase cytochrome P450 (CYP19), in granulosa cells (Raju *et al.*, 2013). While LH is not secreted in high concentrations during the early follicular phase (Reed and Carr, 2000), low concentrations of LH are sufficient to activate the LH receptor (LH-R) in proliferated theca cells, thereby activating the cAMP-mediated PKA pathway and stimulating the conversion of pregnenolone to P4 (Gervásio *et al.*, 2014; Taraborrelli, 2015). Subsequently, P4 is converted into testosterone (T) in the theca cells (Miller and Auchus, 2011; Gervásio *et al.*, 2014; Taraborrelli, 2015). T diffuses from the theca cells into granulosa cells, where CYP19 converts it into E2 (Raju *et al.*, 2013; Gervásio *et al.*, 2014). This dual action of LH and FSH on different cell layers is often referred to as the 'twocell, two-gonadotrophin' model (Raju *et al.*, 2013; Amstrong *et al*., 1979). Consequently, the production of E2 creates a negative feedback loop exerted on the pituitary gland and thus suppresses FSH expression and secretion (Reed and Carr, 2000). The follicle that can withstand this reduced FSH stimulation while maintaining an E2-dominant environment, will continue to mature (Reed and Carr, 2000; Miller and Auchus, 2011). However, other follicles will not develop further under low FSH stimulation and will undergo atresia (Reed and Carr, 2000). The dominant follicle is equipped with sufficient FSH-R to sustain CYP19 expression even under low FSH concentrations and thus continues to produce E2 (Miller and Auchus, 2011). In the mid-follicular phase, the E2 concentrations rise sufficiently to trigger a positive feedback mechanism on the hypothalamus and pituitary gland to promote LH secretion (Reed and Carr, 2000). This ultimately leads to the LH surge that is necessary for ovulation (Reed and Carr, 2000). This highlights the close connection between folliculogenesis and steroidogenesis.

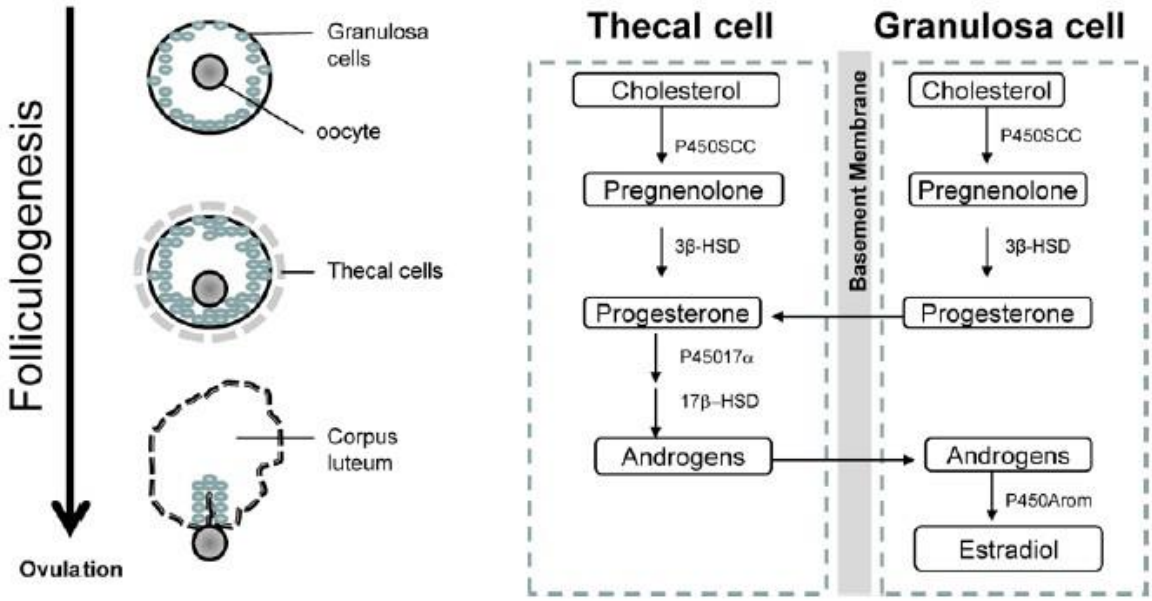


Figure 1.4: A representation of E2 synthesis in the theca and granulosa cells of the follicle, during folliculogenesis. *The follicle is made up of theca and granulosa cells. During folliculogenesis, these cellular layers will proliferate under the stimulation of LH and FSH. LH binds to its receptors in the theca cells and increases the expression of the steroidogenic enzymes. Therefore, this leads to the synthesis of androgens. Concurrently, FSH stimulates the expression of aromatase (CYP19) in the granulosa cells. Subsequently, the androgens will diffuse from the theca cells into the granulosa cells and be converted into E2 by aromatase* (Cui, J. Shen, Y. and Li, 2013).

1.3.1.2 E2 synthesis during the luteal phase

Following the follicular phase and ovulation, the granulosa cell layer of the follicle enlarges, accumulates lutein, and merges with the theca cell layer (Reed and Carr, 2000). The merge of the granulosa and theca cell layers forms the corpus luteum (Reed and Carr, 2000). Although the different cellular layers of the pre-luteinized follicle merge, there remains some distinction in the cell types of the corpus luteum (Devoto *et al.*, 2002). In humans, the paraluteal cells originate from theca cells, while the true luteal cells originate from granulosa cells (Sanders and Stouffer, 1997; Devoto *et al.*, 2002). Interestingly, during the luteal phase, E2 synthesis still occurs in both of these two cell types. The para-luteal cells produce androgens, which are subsequently aromatized into estrogen in the true luteal cells (Devoto *et al.*, 2002).

Similar to the roles of the theca and granulosa cells in E2 synthesis during the follicular phase. However, the key difference during the luteal phase is that LH is the main driver of

steroidogenesis (Devoto *et al.*, 2002). Given the E2 negative feedback on FSH expression and secretion at both the hypothalamus and pituitary level, there is no FSH stimulus for CYP19 expression in the true luteal cells (Reed and Carr, 2000; Devoto *et al.*, 2002). LH takes over the regulation of CYP19 mRNA and protein synthesis in the granulosa-derived true luteal cells (Devoto *et al.*, 2002). LH subsequently continues to increase the expression of CYP19 and other enzymes involved in androgen synthesis, in the para-luteal cells to produce E2 and P4 (Devoto *et al.*, 2002).

1.4 Regulators of LH and FSH

LH and FSH can be regulated at multiple levels, including regulation of transcription and post-transcriptional regulation such as regulation of protein synthesis and secretion (Burger *et al.*, 2004a). A variety of hormones, including GnRH, activin A, inhibin B, follistatin, glucocorticoids, androgens, and P4, have been shown to regulate LH and FSH levels (Burger *et al.*, 2004a).

1.4.1 Regulation of LH and FSH by GnRH

GnRH is secreted from the hypothalamus in a pulsatile action (Acevedo-Rodriguez *et al.*, 2018; Marques *et al.*, 2022). This refers to the episodic release of GnRH in pulses (Marques *et al.*, 2022). GnRH pulses are characterized by their amplitude and frequency (Ellis and Marshall, 1989; Stamatiades and Kaiser, 2018). The amplitude of GnRH pulses refers to the height of the GnRH pulse, which relates to the dose of GnRH released in a single pulse (Ellis and Marshall, 1989; Marques, 2022). The frequency refers to the number of GnRH pulses secreted from the hypothalamus in a given time frame (Ellis and Marshall, 1989). Both the frequency and amplitude of GnRH pulses play a role in the differential regulation of LH and FSH (Casteel and Singh, 2022). Additionally, GnRH regulates LH and FSH at a transcriptional and secretory level (McNeilly *et al.*, 2003; Thackray, Mellon and Coss, 2010).

At a transcriptional level, the binding of GnRH to its receptor (GnRH-R) activates multiple protein kinase pathways and transcription factors involved in regulating transcription of Cga, LHβ and FSHβ mRNA expression (Fowkes, King and Burrin, 2002; Ciccone *et al.*, 2010; Thackray, Mellon and Coss, 2010; Mistry *et al.*, 2011; Das and Kumar, 2018; Stamatiades and Kaiser, 2018). Interestingly, LHβ and Cga mRNA levels are upregulated at higher frequencies of GnRH pulses, while FSHβ is upregulated at lower frequencies (Haisenleder *et al.*, 1991; Kaiser *et al.*, 1997; Stamatiades and Kaiser, 2018; Marques *et al.*, 2022). In murine primary pituitary cell culture and ovariectomized rats, a higher amplitude in GnRH pulses results in higher LHβ and FSHβ mRNA levels. However, the dose of GnRH in a single pulse does not change the levels of Cga mRNA (Haisenleder *et al.*, 1991; Kaiser *et al.*, 1997).

In addition, there is a positive correlation between the frequency of GnRH pulses and the levels of GnRH-R mRNA. This in turn will affect the mRNA levels of Cga, LHβ, and FSHβ (Turgeon *et al.*, 1996; Kaiser *et al.*, 1997). Increased frequencies of GnRH pulses result in elevated levels of GnRH-R mRNA, while lower frequencies of GnRH decrease GnRH-R mRNA levels (Turgeon *et al.*, 1996; Kaiser *et al.*, 1997). Thus, it appears that GnRH plays a role in modulating the mRNA levels of GnRH-R to regulate its activity, which is necessary for the upregulation of Cga, LHβ and FSHβ transcription (Turgeon *et al.*, 1996; Kaiser *et al.*, 1997). These intricate mechanisms underline the sophisticated regulation of gonadotropin subunit genes by GnRH.

The trend of high-frequency GnRH pulses increasing LHβ mRNA levels is maintained at the level of LH protein secretion (Nicol *et al.*, 2004). Interestingly, the amount of LH protein secreted does not equate to the amount of LHβ transcribed (McNeilly *et al.*, 2003). This is largely due to the fact that LH protein is pre-packaged into granules (McNeilly *et al.*, 2003; Nicol *et al.*, 2004). The pre-packing of LH into granules allows the release of LH at different time points and in different amounts (McNeilly *et al.*, 2003; Nicol *et al.*, 2004). Key granin proteins, such as secretogranin II (SgII) are required for the formation of LH granules (Nicol *et al.,* 2004). Interestingly, GnRH stimulation in the mouse pituitary gonadotrope cell line (LβT2) and in rats caused increased SgII and LH secretion, while in the absence of GnRH, Sgll was not secreted and only basal LH protein secretion was observed (Nicol *et al.*, 2004). These results indicate the role GnRH plays in stimulating the aggregation of LH granules through the elevation of SgII levels, thereby priming LH secretion (Nicol *et al.*, 2004). Thus, these results

show that GnRH stimulates both LHβ expression and LH protein secretion and employs different mechanisms in order to upregulate LH transcription and secretion. However, the mechanism behind GnRH regulation of SgII secretion is still unclear. It is important to note that these studies were mainly performed in sheep, rats and a mouse cell line; therefore, it is unclear whether this mechanism is species-specific or more generalized.

In contrast to LH, GnRH mostly regulates FSH at a transcriptional level (transcription of the FSHβ gene) and not by regulation of FSH protein secretion (Das and Kumar, 2018). Therefore, the rate of FSH secretion is linked to FSHβ transcription (McNeilly, 1988). A study in women found that a GnRH antagonist decreased FSH secretion by only 16% (Hall *et al.*, 1988). The authors concluded that there may be other GnRH-independent mechanisms more crucial for FSH secretion (Hall *et al.*, 1988). Additionally, in sheep and in rat pituitary cells, hypothalamic neuropeptides increased the sensitivity of GnRH receptors, which resulted in higher GnRH-induced FSH secretion (McNeilly, 1988; Sawangjaroen *et al.,* 1997). These studies showcase that there are additional factors that either together with or independently of GnRH, increase FSH secretion. Thus, this further highlights the fact that the majority of direct GnRH regulation of FSH occurs mainly at the transcriptional level rather than at the protein secretion level.

GnRH plays a distinct role in regulating the secretion of gonadotropins, with a more direct impact on LH protein production compared to FSH protein secretion. While GnRH plays a crucial role in regulating gonadotropin hormones, it is not the sole factor responsible for regulating the gonadotropin hormones. Other hormonal regulators of LH and FSH also play a part in the differential regulation of both LH and FSH gene expression and protein secretion.

1.4.2 The role of activin, inhibin and follistatin in LH and FSH regulation

The peptide hormones activin A and B, inhibin B, and follistatin, play a significant role in the regulation of LHβ and FSHβ gene expression and secretion. Activins stimulate, while inhibin B and follistatin inhibit LHβ and FSHβ gene expression and secretion (Burger *et al.*, 2004a). Additionally, activin upregulates GnRH-R mRNA in gonadotropes, which increases GnRH sensitivity and subsequent GnRH-induced LHβ and FSHβ transcription and secretion in murine

primary pituitary cells (Gregory and Kaiser, 2004). Both activins and inhibin B are expressed in the ovaries and gonadotropes (Burger *et al.*, 2004a; Das and Kumar, 2018). Follistatin is a glycoprotein that is expressed in most pituitary cell types (Burger *et al.*, 2004a; Bilezikjian *et al.*, 2012; Das and Kumar, 2018). Inhibin B blocks activin activity by competitive binding to the type II activin receptor (ACT-RII) (Gregory and Kaiser, 2004; Das and Kumar, 2018). Follistatin directly binds to the C-terminus of activin, which is required for activin to bind to ACT-RII. Thus, follistatin neutralizes stimulation of both LHβ and FSHβ transcription by activin (Gregory and Kaiser, 2004; Bernard *et al.*, 2010).

1.4.3 The role of steroid hormones and their receptors in the regulation of LH and FSH expression and secretion

Steroid hormones modulate targeted gene expression via the binding and activation of steroid hormone receptors (SRs) (Africander, Verhoog and Hapgood, 2011). SRs are ligand activated transcription factors that regulate transcription of target genes (Africander, Verhoog and Hapgood, 2011). There are different members of the SR family, including the androgen receptor (AR), mineralocorticoid receptor (MR), estrogen receptor (ER), glucocorticoid receptor (GR) and progesterone receptor (PR) (**Figure 1.5 A**) (Hapgood, Kaushic and Hel, 2018). Once steroid hormones bind to their cognate SR, the SRs can modulate gene expression via binding to hormone response elements (HRE) in the promoters of the specific genes (Africander, Verhoog and Hapgood, 2011). These HREs are androgen-response elements (ARE), mineralocorticoid-response elements (MRE), estrogen-response elements (ERE), progesterone-response elements (PRE) and glucocorticoid-response elements (GRE) (Africander, Verhoog and Hapgood, 2011). It is important to note that the different SRs bind to their cognate HRE but can also bind to another HRE. For example, all SRs (except ER) can bind to the GRE (Africander, Verhoog and Hapgood, 2011).

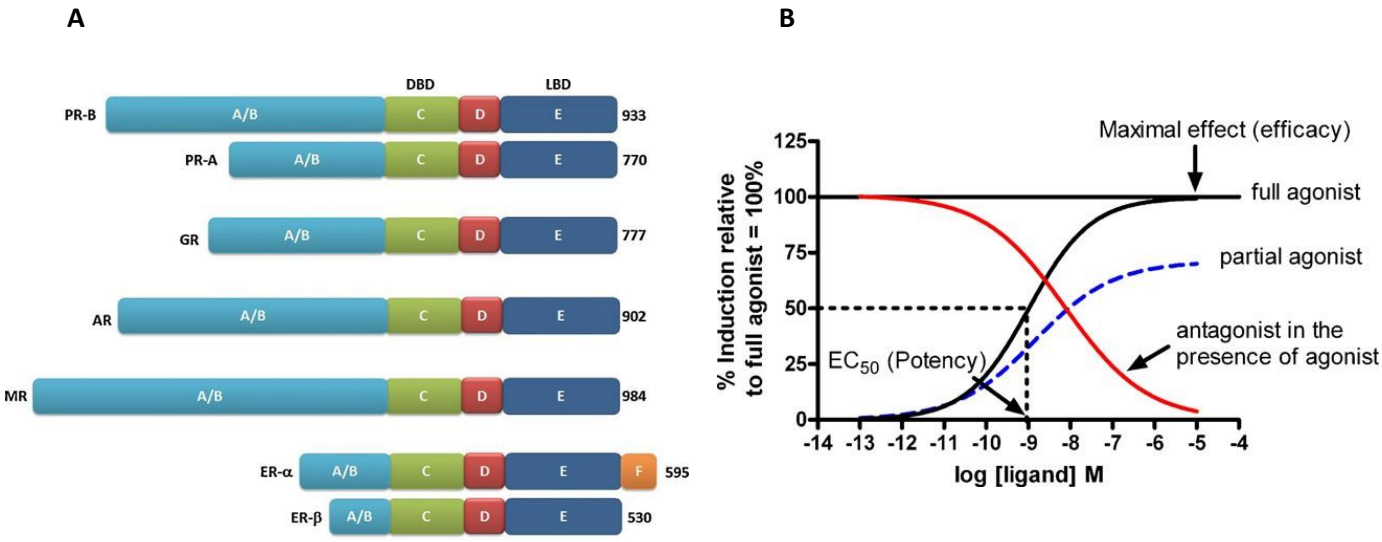


Figure 1.5: Representation of the protein domains of the SRs and a dose-response analysis. *In **A,** the SRs are made up of 3 domains. The first domain (A/B) is the transaction domain, the second domain is made up of the DNA- binding domain (C) and the hinge region (D). The last domain is the ligand binding domain (E). The ER has a distinct carboxy terminal (F)* (Africander, Verhoog and Hapgood, 2011). *In **B**, an example of a dose-response analysis is represented and showcases the relative gene response of a full agonist, partial agonist and an antagonist in increasing concentrations of the ligand measured. The efficacy and EC_{50} (potency) are also displayed* (Africander, Verhoog and Hapgood, 2011).

SRs are involved in the mediation of multiple cellular processes. For example, the GR is known for its immunomodulatory effects when bound to either endogenous cortisol or synthetic analogues of cortisol (De Bosscher, Vanden Berghe and Haegeman, 2003; Hapgood *et al.*, 2014). Theoretically, ligands bind specifically to their respective SRs (Hapgood *et al.*, 2014). However, due to the similarity of the protein structure of the ligand binding domains of these SRs, there are crossovers in ligands binding to other off-target SRs (Hapgood, Kaushic and Hel, 2018). In most cases, the ligands can bind to other SRs but cannot induce the same effects as the intended ligand for the SR (Hapgood, Kaushic and Hel, 2018). On the other hand, there is evidence that some ligands are able to act as partial or full agonists, thereby partially or fully activating off-target SRs to exert effects on different cellular processes (**Figure 1.5**) (Hapgood, Kaushic and Hel, 2018). There are various steroid hormones and SRs that have been shown to regulate LH and FSH expression and protein secretion (Burger *et al.*, 2004a; Thackray, Mellon and Coss, 2010; Das and Kumar, 2018).

1.4.3.1 Glucocorticoids

Glucocorticoids are produced via the hypothalamic-pituitary-adrenal (HPA) axis (Sheng *et al.*, 2021). The HPA axis is a neuroendocrine network consisting of the paraventricular nucleus of the hypothalamus, the anterior pituitary gland, and the adrenal gland and maintains homeostasis in stress response, primarily by controlling the production of glucocorticoids from the adrenal gland (Smith and Vale, 2006; Sheng *et al.*, 2021). The hypothalamus releases corticotropin releasing factor (CRF) and vasopressin (VPN) from the hypophysiotropic neuron (Smith and Vale, 2006; Sheng *et al.*, 2021). CRF and VPN bind to their respective receptors in the anterior pituitary gland and subsequently prompt these cells to produce and release adrenocorticotropic hormone (ACTH) (Smith and Vale, 2006; Sheng *et al.*, 2021). ACTH then enters the bloodstream and binds to the melanocortin type 2 receptor in the adrenal gland, stimulating the production of glucocorticoids (Smith and Vale, 2006; Sheng *et al.*, 2021). Similar to the HPO axis, the HPA axis is also regulated by intra-axis feedback mechanisms. Additionally, both the HPG and HPA axes regulate each other (Acevedo-Rodriguez *et al.*, 2018). For example, glucocorticoids from the HPA axis regulate FSH and LH gene expression.

There are very few studies that have investigated LH and FSH regulation by glucocorticoids in women (Warren, Siris and Petrovich, 1977; Huerta *et al.*, 1995; Woods, Mitchell and SmithDiJulio, 2009; Schliep *et al.*, 2015). One study demonstrated that severe illness and traumatic brain injury led to increased cortisol levels and subsequent reduced plasma LH and FSH levels in post-menopausal women (Warren, Siris and Petrovich, 1977). However, other studies suggest that high stress correlates with high FSH plasma concentrations (Huerta *et al.*, 1995; Woods, Mitchell and Smith-DiJulio, 2009; Schliep *et al.*, 2015). There are a larger number of studies conducted in animal models. In ovariectomized mice, glucocorticoids have differential effects on LH and FSH at the pituitary (McAndrews *et al.*, 1994; Breen *et al.*, 2012). Studies show that corticosterone decreases LHβ mRNA levels, while both corticosterone and cortisol increase FSHβ mRNA levels and FSH serum levels in LβT2 cells and female rats (McAndrews *et al.*, 1994; Breen *et al.*, 2012). Interestingly, glucocorticoids suppress both LH and FSH at a hypothalamic level in mice (Iwasa *et al.*, 2017). Cortisol elevated the endogenous GnRH antagonist, RFamide-Related Peptide-3 (RFRP3) in mice, which is released from neurons

in the hypothalamus (Iwasa *et al.*, 2017). This increase in RFRP3 by cortisol consequently blocked the GnRH-induced increase in LH and FSH protein secretion (Iwasa *et al.*, 2017). In rodents, cortisol thus appears to exert differential effects on FSH levels at the pituitary and hypothalamic levels, via the involvement of RFRP3. In sheep, injected cortisol levels reduced LH secretion pulse frequency, while both injected cortisol and elevated endogenous cortisol (induced by psychosocial stress due to isolation) inhibited the pre-ovulatory LH and FSH surges in the follicular phase (Breen *et al.*, 2005; Wagenmaker *et al.*, 2010).

The in vitro and ex vivo research in the LβT2 murine pituitary cell line and in primary murine pituitary cell cultures provides more evidence of the mechanism behind the regulation of LH and FSH by glucocorticoids. Corticosterone (the more metabolically active glucocorticoid in mice) decreased LHβ mRNA levels and promoter-reporter activity in mouse primary pituitary and LβT2 cells (Breen *et al.*, 2012). Additionally, the synthetic glucocorticoid dexamethasone (DEX) suppressed GnRH-induced LHβ promoter activity in vitro (Breen *et al.*, 2012). Both GR mRNA and protein are expressed in rat primary pituitary cells and in LβT2 cells (Yokote, Hlsano and Daikoku, 1991; Turgeon *et al.*, 1996; Kotitschke *et al.*, 2009). Chromatin immunoprecipitation (ChIP) assays showed that the endogenous GR co-localized with the LHβ promoter exclusively in the presence of both GnRH and DEX, showing that the GR is involved in the mediation of DEX suppression of GnRH-induced LHβ expression (Breen *et al.*, 2012). Additionally, the GR blocked the binding of the GnRH-activated transcription factor, early growth response factor 1 (Egr-1), which is essential for LHβ transactivation (Breen *et al.*, 2012). Taken together, this study indicated that LHβ gene transcription is suppressed by glucocorticoids, an effect that is mediated by the GR in rodents (Breen *et al.*, 2012). In animal models, glucocorticoids appear to predominantly increase FSHβ expression due to their direct effects on the pituitary (Ringstrom *et al.*, 1992; McAndrews *et al.*, 1994; Thackray, McGillivray and Mellon, 2006). Similarly, in LβT2 cells, DEX increased FSHβ promoter activity (Thackray, McGillivray and Mellon, 2006). This effect is mediated by the GR (Thackray, McGillivray and Mellon, 2006). Furthermore, DEX and activin A synergistically increased FSHβ mRNA levels and FSH protein secretion in LβT2 cells (McGillivray *et al.*, 2007). Moreover, glucocorticoid treatment of primary rat pituitary cells in vitro resulted in increased FSH protein secretion (Ringstrom *et al.*, 1992; McAndrews *et al.*, 1994).

In summary, most reports show that glucocorticoids inhibit both basal and GnRH-induced LH expression and secretion, and this appears to be consistent across non-human mammals and different model systems (Warren, Siris and Petrovich, 1977; Breen *et al.*, 2005, 2012; Wagenmaker *et al.*, 2010; Iwasa *et al.*, 2017). Although LH is also suppressed by cortisol in women, this finding was from one study (Warren, Siris and Petrovich, 1977). There may be factors that confound this result, and thus it cannot be concluded whether the suppression of LH by glucocorticoids is consistent across all species. In contrast to LH, cortisol may have opposing effects on FSH levels. Cortisol increases and decreases FSH in different species and even within specific species, such as humans and rodents (Thackray, McGillivray and Mellon, 2006; Schliep *et al.*, 2015). While it is clear that glucocorticoids can regulate LH and FSH levels, there is very limited information on gonadotropin regulation by cortisol in women, and more research on this topic is needed.

1.4.3.2 Androgens

Androgens, more specifically T, are necessary for the synthesis of E2 and, together with FSH, maintain follicular development (Gervásio *et al.*, 2014). While T concentrations change during the different phases of the menstrual cycle in pre-menopausal women, literature suggests that T concentrations in normally cycling women do not result in feedback on the pituitary gland to regulate LH or FSH gene expression or secretion (Mathor *et al.*, 1985; Miller and Auchus, 2011; Gervásio *et al.*, 2014; Skiba *et al.*, 2019). However, in conditions such as polycystic ovarian syndrome (PCOS) that cause hyperandrogenism in women, there are changes in the normal LH and FSH concentration ranges (Suresh and Vijayakumar, 2015). A hyperandrogenic state in women is associated with a higher LH:FSH ratio, in which serum levels of LH are increased while FSH serum levels are decreased (Suresh and Vijayakumar, 2015). Evidence in women suggests that higher androgen concentrations affect the functions of LH and FSH at the ovarian level and not the synthesis or secretion of these gonadotropins at the pituitary level (Vendola *et al.*, 1999; Gervásio *et al.*, 2014; Suresh and Vijayakumar, 2015). It is also important to note that there are no studies regarding the regulation of gonadotropins in the pituitary or hypothalamus by androgens at low or normal concentrations in women (Bernard *et al.*, 2010; Gervásio *et al.*, 2014). Although the causal pathway for these

changes in LH and FSH levels in PCOS women has not been established, data from in vitro experiments indicate a direct role of increased androgens in regulating LH and FSH levels.

In rat primary pituitary and LβT2 cells, high concentrations of T and dihydrotestosterone (DHT) decreased GnRH-induced LHβ transcription and LH secretion from primary mouse pituitary cells (Curtin *et al*., 2001; Burger *et al*., 2004b; Wang *et al.*, 2019). These inhibitory effects of DHT are mediated by endogenous AR protein (Wang *et al.*, 2019), which is also expressed in rat primary pituitary cells and LβT2 cells (Thackray, McGillivray and Mellon, 2006; O'Hara *et al.*, 2015). In LβT2 cells, an increase in FSHβ promoter activity by DHT was mediated by overexpressed AR, which was directly bound to ARE sites in the FSHβ promoter (Burger *et a*l., 2004b; Thackray, McGillivray and Mellon, 2006). Another study found that androgens suppressed FSH secretion from mice primary pituitary cells (Low, 1995; Bernard *et al.*, 2010). In contrast to mice, T decreases FSHβ mRNA levels while having no effect on FSH secretion in primary pituitary cells from rhesus macaques (Fingscheidt *et al.*, 1998; Bernard *et al.*, 2010). These limited studies indicate potential species-specific differences in the regulation of FSHβ transcription by androgens in primates (Low, 1995; Fingscheidt *et al.*, 1998; Bernard *et al.*, 2010).

In summary, in humans, high androgen concentrations are related to high LH and low FSH concentrations. It is unclear whether androgens regulate LH and FSH synthesis and secretion on a pituitary level or rather affect the functions of LH and FSH in the ovaries in humans. The research done in rodents shows that high concentrations of androgens activate the AR in the pituitary to decrease GnRH-induced LHβ transcription and secretion while increasing FSHβ transcription. It is important to note that there are species-specific differences in androgen regulation of FSHβ transcription and secretion, as shown in primate studies (Fingscheidt *et al.*, 1998; Bernard *et al.*, 2010). Lastly, there is a gap in the literature regarding the regulation of the HPO axis by androgens at low concentrations in both animals and women.

1.4.3.3 Progesterone

The main function of P4 during the menstrual cycle is to regulate the luteal phase and support pregnancy in women (Reed and Carr, 2000; Taraborrelli, 2015). P4 has previously been

suggested to suppress LH and FSH in women via a negative feedback loop at the hypothalamus (Messinisi, 2006). In women, the effect of P4 on gonadotropins is limited, particularly for FSH secretion. While some studies suggest that increased P4 increases LH levels in women (Couzinet *et al.*, 1992; Hutchens *et al.*, 2016), others have shown that increased P4 decreases LH levels (Soules *et al.*, 1984; Filicori *et al.*, 1986; Nippoldt *et al.*, 1987; Kazem *et al.*, 1996). Only one study in women showed that increased P4 did not change FSH levels (Couzinet *et al.*, 1992). A key point to mention is that in these studies in women, the regulation of gonadotropins by P4 occurs in the presence of E2. Differences between the abovementioned studies are possibly due to differences in P4 dosage (concentration), the stage of the menstrual cycle before P4 dosage, as well as the hypothalamic deficiency and thereby lack of hypothalamic feedback in the Couzinet study.

Multiple animal studies consistently show a suppressive effect on LH secretion by P4 (Goodman *et al.*, 1981; Wildt *et al.*, 1981; Ireland and Roche, 1982). However, inconsistent results on the effect of P4 on FSH have been reported in other animal studies (Goodman *et al.*, 1981; Wildt *et al.*, 1981; Ireland and Roche, 1982; Lesoon and Mahesh, 1992). In cattle and monkeys, P4 has no effect on FSH secretion (Wildt *et al.*, 1981; Ireland and Roche, 1982). However, in ewes, P4 enhances E2 suppression of FSH secretion at the pituitary, while in rats, the type of effect on FSH secretion by P4 may be due to the duration of P4 exposure (Goodman *et al.*, 1981; Lesoon and Mahesh, 1992).

Extensive research has been done to study the direct transcriptional and post-transcriptional effects of P4 on LH and FSH ex vivo and in vitro in both primary pituitary cell cultures and the LβT2 cell line, respectively (Shupnik, Gharib and Chin, 1989; O'Conner *et al.*, 1999a; Thackray, McGillivray and Mellon, 2006; Thackray *et al.*, 2009). P4 decreases GnRH induction of LHβ transcription in LβT2 cells, and this is mediated by endogenous PR-B protein (Thackray *et al.*, 2009) which is expressed in LβT2 cells (Turgeon and Waring, 2006; An *et al.*, 2009). Similarly, on a post-transcriptional level, P4 decreased LHβ mRNA stability in E2-primed rat pituitary cells (Thackray, Mellon and Coss, 2010). Interestingly, P4 alone does not change LH secretion but rather only increases the type of feedback mechanism on LH secretion by E2, either at the pituitary or hypothalamus (Goodman *et al.*, 1981; Wildt *et al.*, 1981; Ireland and Roche, 1982). In contrast to LHβ, P4 increases FSHβ transcription via direct binding of overexpressed PR-B at the PRE site in both the mouse and ovine promoters in rat primary pituitary cells (O'Conner

et al., 1999b; Bernard *et al.*, 2010). In E2-primed rat primary pituitary cells, P4 increased GnRH-induced LH secretion and FSH secretion during a shorter stimulation time, while the opposite effect occurred during a longer stimulation time (Lesoon and Mahesh, 1992). The mechanism behind these varied P4 effects due to P4 exposure time appears to involve E2 priming of PR-B synthesis in the pituitary, as suggested in other models (Calderon, Muldoon and Mahesh, 1987; Brann, Putnam and Mahesh, 1991; Lesoon and Mahesh, 1992). Additionally, suppressed LH secretion by P4 at the hypothalamus appears to be consistent in multiple species (Goodman *et al.*, 1981; Wildt *et al.*, 1981; Ireland and Roche, 1982) including women (Soules *et al.*, 1984; Messinisi, 2006). While P4 regulation of both LH and FSH secretion at the pituitary is dependent on exposure time in mice (Lesoon and Mahesh, 1992), this effect has not been investigated in other species.

In summary, P4 regulation of gonadotropins appears to be dependent on the presence of other hormones, such as GnRH, in women (Nippoldt *et al.*, 1987) and animals (Thackray *et al.*, 2009; Bashour and Wray, 2012). In both women and animal studies, E2 is important for P4 regulation of gonadotropin secretion (Goodman *et al.*, 1981; Wildt *et al.*, 1981; Ireland and Roche, 1982; Soules *et al.*, 1984; Calderon, Muldoon and Mahesh, 1987; Couzinet *et al.*, 1992; Lesoon and Mahesh, 1992; Messinisi, 2006; Thackray, Mellon and Coss, 2010; Hutchens *et al.*, 2016). Data from animal studies suggest there may be species-specific differences at both the hypothalamus and pituitary involved in the regulation of FSH by P4 (Goodman *et al.*, 1981; Wildt *et al.*, 1981; Ireland and Roche, 1982; Lesoon and Mahesh, 1992). Furthermore, there are limited studies on P4 regulation of FSH in women (Couzinet *et al.*, 1992; Hutchens *et al.*, 2016). The effects of P4 on the pituitary gonadotropin hormones appear to be dependent on many factors.

1.5 The regulation of HPO axis hormones by DMPA-IM and NET-EN

Currently, it is widely accepted that both DMPA-IM and NET-EN prevent pregnancy by inhibiting ovulation due to a lack of E2 and LH surges (Goldzieher *et al.*, 1970; Mishell *et al.*, 1972; Fraser and Weisberg, 1981). Additionally, there are some studies that show NET-EN and, to a lesser extent, DMPA-IM, prevent follicular maturation (Ortiz *et al.*, 1977). Researchers found that the prevention of follicular maturation correlates with a lack of the mid-cycle E2

peak (Ortiz *et al.*, 1977). Recently the randomized WHICH clinical trial compared E2 levels in 521 pre-menopausal women randomized equally to DMPA-IM and NET-EN and found that both contraceptives caused hypoestrogenism to a similar extent (Singata-Madliki *et al.*, 2024). Additionally, these E2 levels are within the post-menopausal range (**Table 1.1**). This data is the most robust to date. Previous studies show E2 levels in DMPA-IM users are mostly within the E2 range found during the early follicular phase of pre-menopausal women (**references are in Addendum A Table A1 and A2**). However, some studies reported that DMPA-IM suppresses E2 to levels lower than early follicular phase and comparable to postmenopausal E2 levels (**Table 1.1**) (Miller *et al.*, 2000; Clark *et al.*, 2001;Hickey, Marino and Tachedjian, 2016).While some reports do show NET-EN maintaining E2 levels within an early follicular phase (Goebelsmann *et al.*, 1979; Saleh *et al.*, 1983), these studies are not as robust as another study (Lawrie *et al.*, 1998) (**Table 1.1**). More recent studies do indicate a larger E2 suppression in both DMPA-IM and NET-EN users compared to women not on any hormonal contraception (Lawrie *et al.*, 1998; Hickey, Marino and Tachedjian, 2016; Dabee *et al.*, 2019)**.** While there are multiple explanations for the different results obtained in the previous studies before the WHICH trial, such as low power, sampling time, demographic characteristics, and/or different methodologies for E2 determination (Mishell *et al.*, 1968, 1972; Jeppsson and Johansson, 1976; Toppozada, Parmar and Fotherby, 1978; Goebelsmann *et al.*, 1979; Saleh *et al.*, 1983). Additionally, E2 levels may vary depending on the number of injections and the time after injection (Jeppsson and Johansson, 1976; Toppozada, Parmar and Fotherby, 1978; Goebelsmann *et al.*, 1979; Saleh *et al.*, 1983). Furthermore, a key limitation in most studies, including the WHICH trial, was that women who may have had non study progestins in their serum at the time of E2 measurements were not excluded. Although the WHICH trial did produce robust data on E2 levels in women on DMPA-IM and NET-EN, the mechanism by which DMPA-IM and NET-EN cause hypoestrogenism is still not known.

As previously discussed, LH and FSH are important regulators for E2 synthesis (Raju *et al.*, 2013). Most of the literature shows DMPA-IM and NET-EN prevent LH and FSH surges (**Addendum A, Table A1**). LH levels reported in DMPA-IM and NET-EN users are quite consistent between studies and are usually within the early follicular phase (Jeppsson and Johansson, 1976; Jeppsson *et al.*, 1977; Goebelsmann *et al.*, 1979; Jeppsson *et al.*, 1982; Saleh *et al.*, 1983) or luteal phase (Mishell *et al.*, 1972; Ortiz *et al.*, 1977). Similarly, FSH levels are mostly reported to be within the early follicular phase or luteal phase in DMPA-IM and NET-

EN users (Mishell *et al.*, 1972; Jeppsson and Johansson, 1976; Jeppsson *et al.*, 1977; Goebelsmann *et al.*, 1979; Jeppsson *et al.*, 1982). However, there are some studies that do report differences in the effect of DMPA-IM on FSH. In one study, FSH levels were found to be decreased in women on DMPA-IM compared to normal cycling women (Perez-Lopez, L'Hermite and Robyn, 1975), while another study found that FSH levels increased in women on DMPA-IM (Siregar, Rita and Yusrawati, 2019). Many of these studies have significantly low power due to their small study populations (1-33 women) (Perez-Lopez, L'Hermite and Robyn, 1975; Jeppsson *et al.*, 1982; Siregar, Rita and Yusrawati, 2019) (**Addendum A, Table A1**). Additionally, some of these studies do not measure statistical significance, correct for confounding factors such as BMI, ethnicity, and the number of injections taken by participants, do not measure for gonadotropins at peak progestin concentrations, and do not account for the time in which participants received the injections relative to their menstrual cycle (Goldzieher *et al.*, 1970; Perez-Lopez, L'Hermite and Robyn, 1975; Jeppsson *et al.*, 1977; Sten Jeppsson *et al.*, 1982). This is important as ovulation is not inhibited in the first 3 months in women who have received a DMPA-IM injection after day 7 of their cycle (Siriwongse *et al.*, 1982; Petta *et al.*, 2001). Therefore, depending on when the injection was given in previous studies, an effect on gonadotropins by DMPA-IM may not be detected or may be different from the effects after only one injection (Siriwongse *et al.*, 1982; Petta *et al.*, 2001). Besides the conflicting data on FSH regulation by DMPA-IM, the mechanism whereby DMPA-IM and NET-EN regulate LH and FSH, as well as the potential causal link thereof with decreased E2, has not been investigated in women, animals or in vitro models. Furthermore, only one non-randomized comparative study between women on injectable progestin contraceptives and on no hormonal contraceptives, showed decreased LH levels. However, this study was underpowered (11 women) (Dabee *et al.*, 2019). Additionally, there are no such studies that compare FSH levels in women on injectable progestin-only contraceptives and women on no hormonal contraceptives.

Given that GnRH is a key regulator of the HPO axis, it is possible that DMPA-IM and NET-EN can regulate gonadotropins and E2 at the hypothalamic level and/or at the pituitary level. However, there are no current studies that measure GnRH concentrations in women on DMPA-IM or NET-EN. Furthermore, the lack of this research opens up the question as to what level of the HPO axis these contraceptives exert their effects on in order to cause hypoestrogenism in women. One study attempted to understand whether injectable progestin contraceptives

would block pituitary responsiveness to GnRH (Toppozada, Parmar and Fotherby, 1978). This was done by measuring LH and FSH secretion in women on DMPA-IM and NET-EN that were injected with exogenous GnRH (Toppozada, Parmar and Fotherby, 1978). The authors concluded that the suppression of the gonadotropins was rescued by
GnRH administration, and thus that the contraceptives most likely act upstream of the pituitary (Toppozada, Parmar and Fotherby, 1978). However, these authors did not take into account that the administration of GnRH could likely reverse the effects of the contraceptives on the gonadotropins. Additionally, other papers have replicated the same experiments but generated inconclusive results that contradict the previous study (Mishell *et al.*, 1972; PerezLopez, L'Hermite and Robyn, 1975). Furthermore, these studies do not take into account the endogenous GnRH that is still present in women, which may confound the results (Mishell *et al.*, 1972; Perez-Lopez, L'Hermite and Robyn, 1975).

Taken together, it is not clear at what level of the HPO axis these contraceptives act to induce hypoestrogenism in women. There is therefore a need for a robust clinical study that directly measures the E2, gonadotropin, and GnRH levels in both DMPA-IM and NET-EN users. While such a study will give information on systemic gonadotropin levels, it will not provide information on the mechanism of regulation of LHβ and FSHβ transcription at the pituitary level.

MPA and NET are synthetic progestins designed to primarily act via the PR to regulate target gene expression (Stanczyk *et al.*, 2013). Given that MPA and NET primarily bind to and activate the PR, it is expected that the progestins will regulate the HPO axis in a similar manner to P4 (as previously discussed in section **1.4.3.3**). However, MPA and NET have also been shown to have off-target effects via the AR (Pérez-Palacois *et al.*, 1983; Africander, Storbeck and Hapgood, 2014). This could result in the progestins acting similarly to androgens in regulating the HPO axis (as discussed in section **1.4.3.2**). Furthermore, MPA, but not NET, has been shown to bind to and act as a partial agonist for the transactivation and as a full agonist for the transrepression of the GR (Ronacher *et al.*, 2009). Thus, MPA may exert glucocorticoid-like effects on the HPO axis (as previously discussed in section **1.4.3.1**). Therefore, depending on the specific steroid receptors present, as well as their relative concentrations in any given system/cell, these hormones could regulate the HPO axis in multiple different ways. Pituitary gonadotropes are reported to express the PR, GR and AR protein in the primary pituitary cells

of rodents, chicken embryos, and non-human primates (Yokote, Hlsano and Daikoku, 1991; Stefaneanu, 1997; Turgeon, Shyamala and Waring, 2001; Bossis *et al.*, 2004; Gordon *et al.*, 2015; O'Hara *et al.*, 2015). Only one study detected AR protein in the human pituitary via an immunocytochemical assay (De Winter, Kimura and Mizokami, 1994). It is important to note that there is limited evidence that shows the presence of GR, PR and AR protein specifically in primary gonadotropes, as primary pituitary cells consist of multiple cell types. Given the possible protein expression of the GR and AR in pituitary gonadotropes, it is likely that there is crosstalk between these off-target SRs and the progestins. Both MPA and NET may act via the AR as well as the PR, while MPA may also act via the GR, in order to exert their effects on gonadotropins (Goebelsmann *et al.*, 1979; Fraser and Weisberg, 1981; Pérez-Palacois *et al.*, 1983). This has also not been investigated in past studies. In summary, addressing these research gaps is imperative for understanding the mechanisms involved in the hypoestrogenic effects exerted by DMPA-IM and NET-EN.

1.6 Summary and thesis rationale

Extensive research has centered on the protective effects of E2 within the FGT, particularly in the context of viral infections such as HIV-1 (Hickey, Marino and Tachedjian, 2016; Hapgood, Kaushic and Hel, 2018). Given the protective effects of E2 in women, an understanding of the mechanisms whereby DMPA-IM and NET-EN decrease E2 may give important insights into potential issues in women's reproductive health, such as the risk of HIV-1 infection. While both contraceptives create a hypoestrogenic environment in women (Hickey, Marino and Tachedjian, 2016; Singata-Madliki *et al.,* 2024), the unknown mechanism(s) behind hypoestrogenism highlights a significant gap in the research on DMPA-IM and NET-EN.

Levels of LH and FSH, key regulators of E2 synthesis, are influenced by DMPA-IM and NET-EN use. While the majority of studies show that DMPA-IM and NET-EN suppress the preovulatory LH and FSH surges and maintain basal LH and FSH levels within ranges similar to the early follicular and luteal phases (Mishell *et al.*, 1972; Jeppsson and Johansson, 1976; Ortiz *et al.*, 1977; Goebelsmann *et al.*, 1979; Jeppsson *et al.*, 1982), there are some contradictory reports of FSH regulation by DMPA-IM (Perez-Lopez, L'Hermite and Robyn, 1975; Siregar, Rita and

Yusrawati, 2019). Many of the past studies do not have robust data for LH and FSH in women on these contraceptives, and only one study compared the regulation of LH by DMPA-IM and NET-EN in parallel for a small number of women (11 women) (Dabee *et al.*, 2019). In addition, no studies measured the regulation of LH and FSH at peak serum progestin concentrations in more than 3 women (Ortiz *et al.*, 1977). Furthermore, there was a large inter-individual variability in the results when LH and FSH regulation were measured at peak MPA concentrations (Ortiz *et al.*, 1977). Despite the crucial role of GnRH in the HPO axis and E2 synthesis, there are no data available on GnRH levels of women on DMPA-IM and NET-EN. Therefore, a robust study is needed to directly measure all the forementioned factors and compare the regulation of the gonadotropins by DMPA-IM and NET-EN.

The recently conducted WHICH clinical trial, where women were randomized equally to DMPA-IM and NET-EN, showed that both DMPA-IM and NET-EN resulted in hypoestrogenism (Singata-Madliki *et al.*, 2024). In a secondary WHICH study (unpublished), the levels of GnRH, LH and FSH were measured in serum from 200 women (DMPA-IM n = 100 and NET-EN n=100) at baseline (Day 0 or D0) and 25 weeks (25W), i.e., one week after the fourth injection for NET-EN and the third injection for DMPA-IM, corresponding to peak serum MPA and NET concentrations (Avenant *et al.*, 2023).

The first part of this thesis will focus on analyzing the within-group (D0 DMPA-IM vs 25W DMPA-IM and D0 NET-EN vs 25W NET-EN) and between-group (change DMPA-IM (25W-D0) vs change NET-EN (25W-D0) differences in the GnRH, LH, and FSH data obtained in the secondary WHICH study. While the effects of DMPA-IM and NET-EN on E2 levels are known for the whole WHICH cohort (521 women), this thesis will also determine the within- and between-group differences on E2 levels in the same subgroup (n = 200) of women in which the GnRH, LH, and FSH levels were determined. This will allow a comparison of the levels of all these hormones for the same participants. Apart from being the first study to report on GnRH levels in women on DMPA-IM and NET-EN, this study will also be the first randomized trial with head-to-head comparisons between DMPA-IM and NET-EN that examined LH, FSH, and E2 levels at peak serum MPA and NET concentrations. In addition, while the WHICH primary paper reported on E2 levels in the whole cohort (521 women), they did not correct for non-study progestins, which could possibly confound the results. In this thesis, all WHICH data on E2, GnRH, LH, and

FSH will also be analyzed after excluding for non-study progestins, thereby eliminating potential confounding effects.

While the analysis of the WHICH clinical samples will give insight into whether these hormones in the HPO pathway are regulated by DMPA-IM and NET-EN, the clinical data alone might not be able to give mechanistic insight into how MPA and NET regulate the hormones of the HPO axis. The second and largest part of this thesis will therefore focus on investigating whether MPA or NET exert direct effects on LHβ and FSHβ transcription, mRNA levels and LH and FSH secretion from the pituitary. In order to test this, a well-established murine pituitary gonadotrope cell line model was used. It was important to use a gonadotrope cell line, as primary pituitary cell cultures contain different cell types, such as gonadotrophs and corticotrophs, that may have paracrine effects and would be a confounding factor when investigating direct effects on gonadotropins at the pituitary level. The mature murine pituitary gonadotroph cell line (LβT2) was chosen as an in vitro model given the fact that these cells have been previously shown to express mRNA and protein for both the common α and unique β subunits of FSH and LH (Thomas P, Mellon PL, Turgeon J, 1996; Daschner *et al.*, 2002). Additionally, the cells respond to GnRH stimulation, due to the expression of the GnRH receptor (GnRH-R) gene and retention of the signalling pathways downstream of the GnRH-R (Windle, Weiner and Mellon, 1990). Furthermore, the LβT2 cells express the relevant transcription factors that are fundamental to the regulation of transcription of the genes for subunits of FSH and LH (Windle, Weiner and Mellon, 1990). The LβT2 cells have also been shown to secrete FSH and LH (Nicol *et al.*, 2004). Additionally, there is no human gonadotrope pituitary cell line available, so LβT2 cells remain the best available cell line for mechanistic pituitary studies.

The steroid ligands and hormones used throughout all experiments were MPA, NET, DEX and P4. DEX is a synthetic glucocorticoid and a full agonist for the GR, while P4 is an agonist for and activates the PR (Africander, Verhoog and Hapgood, 2011). Additionally, it would be expected that MPA would act like DEX and P4, while NET would only act like P4, since both MPA and NET are PR agonists, while MPA is a partial to full agonist for the GR, unlike NET.
Therefore, DEX and P4 were used as controls since their regulation of LHβ and FSHβ via the GR and PR has been previously studied. GnRH is established as an important regulator of the

genes for the α and β subunits of LH and FSH from studies in animal cell models (Burger *et al.*, 2004b). Additionally, GnRH regulates LH and FSH secretion (Nicol *et al.*, 2002). Thus, stimulation with GnRH was used to compare basal and GnRH-induced regulation by MPA and NET.

In order to gain more insight into the role of the different SRs involved in LH and FSH regulation by MPA and NET in the pituitary gonadotroph cells, SR content was investigated by means of western blot analysis. After determining the SRs expressed in the cells, SR-specific antagonists were used to further investigate the role of a specific SR on MPA- and NET-induced LH and FSH regulation. A concentration of 100 nM of all hormones was used. This concentration is physiologically relevant, given that this is the highest Cmax reported for MPA and NET at peak serum levels in women (Bick *et al.*, 2021). DEX and P4 were used at 100 nM to ensure that the GR and PR were saturated by all the steroid ligands to elicit a maximal response (Hapgood *et al.*, 2014). 100 nM GnRH was chosen based on literature showing that this concentration of GnRH resulted in regulation of FSH and LH subunit gene expression and native protein secretion in the LβT2 cells (Nicol *et al.*, 2002).

1.7 Central hypothesis:

DMPA-IM and NET-EN act directly on pituitary gonadotropes to modulate LH and FSH levels.

The aims for this thesis are as follows:

1. **To determine the concentrations of E2, GnRH, LH and FSH in serum of a subgroup of women (n = 193) randomized equally to DMPA-IM and NET-EN.** Data was obtained from serum samples collected from women randomized to DMPA-IM and NET-EN in the WHICH clinical trial, at baseline (before the first contraceptive injection) and at 25W after the first injection, corresponding to peak serum MPA and NET levels. Data was analyzed to determine the median concentrations as well as the change in concentrations for each contraceptive (within group) and between contraceptives (between groups).

2. **To investigate the direct effects of MPA and NET on gene expression of LH and FSH subunits and secretion of LH and FSH proteins in a pituitary gonadotrope model.** The immortalized LβT2 cells were used for all in vitro experiments. Promoter-reporter assays and quantitative real-time polymerase chain reaction (RT-qPCR) were conducted to study the regulation of LHβ and FSHβ and Cga promoter-reporter activity and/or mRNA levels, respectively, by MPA, NET, DEX and P4. Select experiments were performed in the absence and presence of GnRH. Enzyme-linked immunosorbent assays (ELISAs) were performed to measure LH and FSH protein secretion under the stimulation of the selected hormone ligands in the absence and presence of GnRH.

3. **To investigate the involvement of SRs in the regulation of LHβ and FSHβ gene expression by MPA and NET**. In order to study SR involvement, RT-qPCR was first used to measure SRs gene expression in LβT2 cells, and subsequently, a western blot was done to detect SR protein expression. Lastly, SR-specific antagonists in combination with the other hormone ligands were used in promoter-reporter assays and RT-qPCR to measure gonadotropin promoter-reporter activity and mRNA levels.

Chapter 2: Materials and Methods

All experiments on serum samples and subsequent data collection were performed by senior members in the Hapgood lab or external laboratories. The analyses on the data collected from the Women's Health, Injectable Contraception and HIV clinical trial presented in this thesis were performed by the candidate.

2.1 Primary study: WHICH clinical trial

The primary study was registered by the Pan African clinical trial registry, with trial number PACTR 202009758229976. In this open-label, randomized trial, 521 HIV-1 negative women between the ages of 18-40 were recruited from 2 provincial sites in South Africa: 331 participants were recruited from the University of Witwatersrand MatCH research unit (MRU) in KwaZulu Natal, and 189 participants were recruited from the East London and Mdantsane public health clinics and hospitals (Frere and Cecilia Makiwane Hospitals) (ECRU). These women were randomly assigned to either the 3-monthly progestin-only injectable contraceptive DMPA-IM 150 mg intramuscular (n = 262) or the 2-monthly progestin-only injectable contraceptive NET-EN 200 mg intramuscular (n = 259), with equal numbers of women randomized to each contraceptive within sites. Participants were excluded if they were HIV-1 positive. Additionally, women were excluded if they self-reported use of DMPA-IM for 6 months or NET-EN for 4 months before the trial started, and if they self-reported use of any medication that could interfere with the detection of steroid hormones, such as preexposure prophylactic drugs (PrEP). The objectives of the primary study were to investigate the effects of DMPA-IM and NET-EN on depression (measured using Beck's Depression Inventory) and E2 levels (Singata-Madliki *et al.*, 2024). Blood was taken from the participants before the first injection of either DMPA-IM or NET-EN, at baseline time point D0, as well as approximately 7 days after the participants received their 6 month injection, at 25W. This was done to ensure that blood was taken at the Cmax of MPA and NET peak serum levels (Avenant *et al.*, 2023). Over the course of the 6 months, women had 3 injections of DMPA-IM and 4 injections of NET-EN. The primary outcomes as well as sexual behavior data from the trial have recently been published.

The data that was analyzed in this thesis formed part of a secondary study of the WHICH trial. One of the objectives of the secondary study was to determine the systemic concentrations of hormones likely to be involved in the regulation of systemic E2 levels in the WHICH participants. For the secondary study, serum samples from 100 randomly selected participants from each of the MRU and ECRU sites, respectively, were chosen (a total of 100 DMPA-IM users and 100 NET-EN users). After excluding participants that became HIV-1 positive over the course of the trial (n = 5) or became pregnant (n = 2), E2, GnRH, LH and FSH levels were determined in the 193 remaining participants (DMPA-IM n = 99; NET-EN n = 94). Details on the techniques and assays that were used to determine E2, GnRH, LH, and FSH, is given in **Addendum B**.

2.2 Ethics and Biosafety

The primary WHICH trial obtained ethics approval from the Faculty of Health Sciences Human Research Ethics Committee (FHS HREC, M180528) of the University of Witwatersrand Research Ethics Committee and from the East London Hospital Institutional Ethics Committee. Permission to conduct the study was also obtained from the Provincial Departments of Health of the Eastern Cape and KwaZulu Natal. All women provided informed, written consent to authorize study participation and the storage of samples.

Ethical approval of the secondary analysis and storage of the WHICH samples used in this study was provided by the Human Research Ethics Committee at the University of Cape Town (HREC 664/2018). Additionally, the Human Research Ethics Committee at the University of Cape Town approved the use of analyzed data from serum samples collected from participants in the WHICH clinical study in this dissertation research (HREC 007/2022). This research complied with the biosafety guidelines (approval number: 210/2011) and procedures set by the Faculty Biosafety Committee at the University of Cape Town. All researchers, including the candidate, did not have access to information that could identify individual participants during or after data collection. The candidate was also blinded as to which contraceptive was associated with which datum identification code.

The candidate conducted all the experiments and data analyses presented in the methods below.

2.3 Cell culture

The immortalized mouse LβT2 pituitary gonadotrope cell line was kindly gifted by Prof. P.L. Mellon at the University of California, San Diego, USA (Alarid *et al.*, 1996; Graham, Nusser and Low, 1999). The LβT2 cell line originates from a pituitary tumor in transgenic mice caused by targeted oncogenesis that used the promoter sequence from the rat LHβ promoter sequence combined with the protein-coding sequences of the SV40 Tantigen (Tag) oncogen. The LβT2 cells were cultured and maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 10% fetal calf serum (FCS) (Thermo Scientific, South Africa, catalogue no. A5256701) and 1% PenStrep (100 IU/mL of Penicillin and 100 ug/mL of Streptomycin) (Sigma Aldrich, South Africa, catalogue no. P4333) (Full DMEM). The cells were propagated by making a 1:5 dilution of thawed frozen LβT2 cells and DMEM supplemented with FCS and PenStrep. Thereafter, the cells were maintained and passaged every 5 days with a 1:5 dilution. The LβT2 cells are sensitive and thus were not passaged beyond passage number 30 (p30) (Ruf-Zamojski *et al.*, 2019). Cells were maintained in a 90% humidified incubator with 5% CO2 at 37℃ and were regularly tested for mycoplasma, using Hoechst staining and fluorescence microscopy (Freshney, 2002). Only cells that were mycoplasma-free were used for experiments.

2.4 Test compounds and plasmids

The following compounds were purchased from Sigma Aldrich, South Africa: (11β,16α)-9Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione (DEX, catalogue no. D4902) 6α-Methyl-17α-hydroxyprogesterone acetate (MPA, catalogue no. M0250000) 17α ethynyl-19- nortestosterone (NET, catalogue no. BP266), 4-Pregene-3,20-dione (P4, catalogue no. P8783) and (DES-GLY10, D-ALA6)-LH-RH ETHYLAMIDE ACE (GnRH, catalogue no. L4513-1MG), 11β-(4-Dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3one (RU486, catalogue no. M8406). Stock solutions of all hormones, except for GnRH, were made up in absolute ethanol (EtOH) and GnRH was made up in sterile water. Final concentrations

used for all the hormones were 100 nM and 0.1% (v/v) ethanol was used as vehicle controls. The promoter reporter construct used were the mouse FSHβ luciferase (mFSHβ-luc (-1990)). The mFSHβ-luc (-1990) contained 1990 bp (from -1990 to -1) of the mouse FSHβ gene promoter that was cloned into the pGL3 basic vector upstream of the luciferase reporter gene. Additionally, the mouse LHβ luciferase (–232/5 mLHβ-luc) was used as a promoter reporter construct. This construct contained 237 bp (from -235 to +5) of the mouse LHβ gene promoter and cloned into the pA3 vector upstream of the luciferase coding sequence. Both the mFSHβ-luc (-1990) and –232/5 mLHβ-luc promoter reporter constructs were kind gifts from Prof. Daniel Bernard (McGill University, Canada) (Bernard, 2004; Fortin *et al.*, 2009). Human LH and FSH promoter constructs are available and were obtained and an attempt was made in this study to investigate responses to these constructs. However, no responses to any ligands were obtained, with the human constructs (data not shown). The pcDNA3-hGR contained the human GR cloned into the pcDNA3 vector, under the control of the SV40 promoter, and was a gift from D.W. Ray (University of Manchester, UK) (Ray *et al.*, 1999). The pSV-hAR contained the human AR cloned into the pSV vector and was under the control of the SV40 promoter. This expression vector was procured from F. Claessens (University of Leuve, Belgium) (Brinkmann *et al.*, 1989). These expression vectors were used to generate the positive controls for human GR or AR respectively, used in the western blot analysis.

2.5 Plasmid transformation and purification

Plasmids were transformed in *Escherichia coli (E.*coli) DH5α using the heat shock method (Armstrong, 1983). Competent DH5α cells (100 µL) were mixed with 10 ng plasmid and placed on ice for 30 minutes. Thereafter, the DNA and bacteria mixture was incubated at 42℃ for 1 minute and returned to ice for another 2 minutes. Cells were mixed with 900 µL SOC media (Super Optimal Broth SOB media 2% *(w/v)* tryptone, 0,5% *(w/v)* yeast extract, 0,05% *(w/v)* NaCl, 1,86% (w/v) KCL, combined with 2 M $MgCl_2$ and 20 nM glucose) and placed on an orbital shaker at 37℃ for 1 hour at 200 rpm. Transformed cells (100 µL) were plated onto LB-agar plates (1% *(w/v)* tryptone, 0,5% *(w/v)* yeast extract, 1% *(w/v)* NaCl, 1,5% *(w/v)* agar) that contained 100 mg/mL ampicillin (Sigma Aldrich, RSA) (catalogue no. A0166-25G) for the selection of cells containing the respective plasmids. As controls for this selection, untransformed cells were plated on LB-agar plates with 100 mg/mL ampicillin. Day cultures

were made by inoculation of a single colony in 5 µL LB containing 100 mg/mL ampicillin and incubated at 37℃ for 6 hours. Subsequently, 200 µL day culture was inoculated in 200 µL LB containing 100 mg/mL ampicillin and incubated at 37℃ overnight. The Nucleo-bond Xtra Maxi DNA purification kit (catalogue no.740_414.50, Separation Scientific, RSA) was used to purify plasmid DNA from the overnight culture, according to the manufacturer's instructions. The quality of the plasmid DNA was verified using the NanoDrop ND100 (NanoDrop technologies, Wilmington, DE, USA). The size and integrity of the plasmid was verified by 1.25% agarose gel electrophoresis, and the plasmid identity was confirmed by restriction enzyme digests and agarose gel electrophoresis (**Addendum C, Figure S1, Tables S1-S2**).

2.6 Promoter-reporter luciferase assays

LβT2 cells were seeded at a density of 2 x 10^5 in a 24-well plate for both the mFSHβ-luc (-1990) and the -235/5 mLHβ-luc promoter-reporter assays. The cells were grown for 48 hours before transfection with 450 ng of the respective plasmids using XtremeGene 9 (Roche Diagnostics, RSA), according to the manufacturer's instructions. The cells were stimulated for 24 hours with the indicated ligands at their respective concentrations and stimulated with GnRH for the last 6 hours, before cells were harvested. After stimulation, the cells were lysed using 1 X reporter lysis buffer (Promega, South Africa). Luciferase activity in the different reporter assays was measured using the Luciferase assay system (Promega, South Africa) and the Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). The protein concentration of the cell lysates from the reporter assays was measured using a Bradford protein assay (Kielkopf, Bauer and Urbatsch, 2020). The raw luciferase activities were normalized against the protein concentrations.

2.7 RNA isolation, cDNA synthesis and RT-qPCR

LβT2 cells were seeded at a density of 6 x 10^5 in a 12-well plate and grown for 24 hours in full DMEM. The cells were then stimulated with the indicated ligands at their respective concentrations in serum-free DMEM for 24 hours. Thereafter, 400 µL TriReagent (Sigma Aldrich) was added to each well for total RNA isolation. The full volume of each well was transferred into individual microfuge tubes. Following this, 80 µL chloroform was added and

samples were vortexed for 15 seconds then centrifuged at 12 000 rcf for 15 minutes. Subsequently, 180 µL of the aqueous phase was pipetted into a new set of microfuge tubes. In order to precipitate the RNA into a pellet, 200 µL isopropanol was added and the tubes were centrifuged at 12 000 rcf. The RNA pellet was washed with 400 µL 75% ethanol and centrifuged at 10 000 rcf. Following the centrifugation, the pellet was air-dried and resuspended in 10 µL of diethyl pyrocarbonate (DEPC) (Sigma Aldrich, catalogue no. D5758) treated water. The resuspended RNA pellet was re-precipitated using a mixture of 1 µL 3% sodium acetate and 9 µL absolute ethanol. This mixture was stored at -80°C overnight. The RNA pellet was subsequently centrifuged at 17 000 rcf, after which the pellet was washed with 750 µL 75% ethanol and centrifuged at 14 000 rcf. The pellet was air-dried and resuspended again with 10 µL DEPC-treated water. The quality of the RNA was verified using the NanoDrop ND100 (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was validated by 1% agarose formaldehyde denaturing gel electrophoresis and visualized by ethidium bromide (EtBr) (Thermoscientific, South Africa, catalogue no.15585011) staining that fluoresced under UV radiation light. The UV light was connected to a camera in the Gel Doc XR+ Gel Documentation System (Bio-Rad, South Africa).

250 ng RNA was used to synthesize cDNA, using the cDNA synthesis kit (Applied Biosciences, RSA) (Tazi *et al.,* 2010). The cDNA reagents were added to the RNA and the mixture was incubated at room temperature for 10 minutes, then at 37°C for 30 minutes and lastly at 80°C for 5 minutes. RT-qPCR was performed using FastStart SYBR® Green Master (Roche Diagnostics, RSA) on a Corbett RotorGene 3000 RT-qPCR machine. The reaction mixture included: FastStart SYBR® Green Master, forward and reverse primers (See **Table 2.1**), nuclease-free water and cDNA in a final volume of 20 µL. The reactions were amplified using 40 cycles of the following protocol: 95°C for 10 seconds, the respective annealing temperature (**Table 2.1**) for 10 seconds and 72°C for 10 seconds. RotorGene software version 1.7.87 (Qiagen, USA) melt curve analysis was used to validate the correct PCR product amplification. 2% agarose gel electrophoresis was conducted to confirm the correct size of the PCR products (see **Table 2.1**). Initially, glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) was used as the housekeeping gene; however, GAPDH has pseudogenes in mice (Sun *et al.*, 2012). These pseudogenes were cDNA that had been inserted in the genome near transposable elements and transcribed intron less mRNA (Sun *et al.*, 2012; Nevone *et al.*, 2023). Thus, although the GAPDH primers were designed to span exon-exon junctions to avoid

amplification of genomic DNA, the trizol method did not always exclude genomic DNA contamination. Thus, this pseudogene cDNA could be amplified and thus detected in the control without reverse transcriptase. This is showcased in **Addendum C** (**Figure A1.1.2**). Therefore, this made the quantification of gene expression relative to GAPDH unreliable. Thus, the TATA-box binding protein (TBP) was subsequently used as a housekeeping gene to prevent the issues faced with GAPDH (**Addendum C Figure A1.1.1**). The primers used to amplify FSHβ gene expression (**Table 2.1**) were changed after the first 3 repeats used in **Figure 4.2.1**. This was because the LβT2 cell stocks with passage numbers greater than 28 used after these repeats did not express FSHβ. (**Addendum C Figure A1.2.1 A-C**). Therefore, an earlier passage number of the LβT2 cell stocks were used throughout the experiment (**Table 2.1, Addendum C Figure A1.2.1 D-F**). Two different primer pairs were tested for efficiency of FSHβ mRNA detection by RT-qPCR. The second FSHβ primer pair from was found to be the most efficacious and were used in all subsequent experiments (Li,2017). The Delta Delta Ct method was used to analyze the RT-qPCR results (Pfaffl, 2004).

Table 2.1: Primer information for RT-qPCR

Gene of interest	Primer sequence	Product length (bp)	Annealing temperature (°C)	Final concentration (mM)	Reference
FSHβ_1*	FWD: 5'-GTTCAGCTTTCCCCAGAAGA-3' REV: 5'-CCTAGTATAGCAGTAGCCCG-3'	224	58	0.4	Feng, Lawson and Melamed, 2008
FSHβ_2*	FWD: 5'-GTGCGGGCTACTGCTACACT-3' REV: 5'-CAGGCAATCTTACGGTCTCG-3'	111	60	0.4	Li, 2017
LHβ	FWD: 5'-GCCTGTCAACGCAACTCTGG-3' REV: 5'-CAGGCCATTGGTTGAGTCCT-3'	300	58	0.4	Feng, Lawson and Melamed, 2008
Cga	FWD: 5'-GAATATTACCTCGGAGGC-3' REV: 5'- CCTAACGAGAAGAGACTGC-3'	287	45	0.25	Dr A. Kotitschke (unpublished)
TBP	FWD: 5'-TGCACAGGAGCCAAGAGTGAA-3' REV: 5'-TGCACAGGAGCCAAGAGTGAA-3'	132	60	0.25	Masilamani, Loiselle and Sutherland, 2014

GAPDH	FWD: 5'-TTCACCACCATGGAGAAGGC-3' REV: 5'-GGCATGGACTGTGGTCATCA-3'	258	58	0.25	Overbergh *et al.*, 1999
GRα	FWD: 5'-TGCTATGCTTTGCTCCTGATCTG-3' REV: 5'-TGTCAGTTGATAAAACCGCTGCC-3'	299	52	0.3	Thackray, McGillivray and Mellon, 2006
PR-A	FWD: 5'-GGTGGGCCTTCCTAACGAG-3' REV: 5'- GACCACATCAGGCTCAATGCT-3'	121	60	0.3	Turgeon and Waring, 2006
PR-B	FWD: 5'- GGTCCCCCTTGCTTGCA-3' REV: 5'-CAGGACCGAGGAAAAAGCAG-3'	121	60	0.3	Turgeon and Waring, 2006
ERα	FWD: 5'-GTCTGGTCCTGCGAAGGCTGCAA-3' REV: 5'- GCCTTCCAAGTCATCTCTCTGACG-3'	235	60	0.3	Schreihofer, 2000
ERβ	FWD: 5'-GCTGTGATGAACTACAGTGTTCCC-3' REV: 5'-TGGACTAGTAACAGGGCTGGCACA-3'	267	60	0.3	Schreihofer, 2000

* $_{\#1}$: These primers were used in the first 3 repeats in Figure 4.2.1

$_{\#2}$: These primers were used in the last 3 repeats in Figure 4.2.1 and in all samples in Figure 4.2.2

2.8 ELISA

LH secretion in supernatants from LβT2 cells was measured via the Sandwich-ELISA method. A mouse LH ELISA kit (Elabscience Biotechnology Co.,USA, catalogue no. E-EL-M3053) and mouse FSH ELISA kit (Elabscience Biotechnology Co., USA, catalogue no. E-EL-M0511) were used to measure LH or FSH secretion, respectively, as per the manufacturer's instructions. The supernatant samples were diluted 1:2 with the sample dilutant provided by the mouse LH ELISA kit.

2.9 Western blot

LβT2 whole cell lysates were used for the western blot. Cells were plated in a 6-well plate at 6 x 10^5 cell density and grown for 24 hours. The positive controls for GR and AR were made by transfecting 1 µg of the pcDNA3-hGR or pSVAR plasmids into 1 x10^5 COS cells. The PR-B stably transfected MDA-MB-231 cells were used as a positive control for PR-B. The cells were washed in ice cold 1 X PBS. The protein was harvested by adding 50 µL of 1 X sample application buffer (100 mM Tris-Cl, 5% (*v/v*) sodium dodecyl sulphate (SDS), 20% (*v/v)* glycerol, 5% *(v/v)* β-mercaptoethanol and 0.1% *(v/v)* bromophenol blue) to each well, followed by scraping and transfer into microfuge tubes. Samples were boiled at 100 °C for 10 minutes.

For the western blots, 8 µL of the samples were loaded onto 8% SDS polyacrylamide gels, and the protein was separated via electrophoresis at 75 V for 20 minutes then at 110 V for 1 hour 15 minutes, in 1 X running buffer (25 mM Tris-HCl, 250 mM glycine and 0,1% *(v/v)* SDS). The separated protein was transferred onto a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, South Africa, catalogue no. RPN303D) at 180 mA for 1 hour in cold 1 X transfer buffer (25 mM TRIS, 200 mM glycine, 20% *(v/v)* methanol). Subsequently, the membrane was cut and placed in separate containers to allow separate incubation with SR antibody and GAPDH antibody (see **Table 2.2**). Thereafter, the membranes were blocked for 1 hour at 25°C by shaking in 4% *(w/v)* ECL blocking solution (4 g ECL blocking powder (Amersham Biosciences, South Africa, catalogue no. RPN2125) in 100 mL Tris-buffered saline (50 mM Tris-HCl, pH 7.5 and 150 mM NaCl; TBS) containing 0,1% *(v/v)* Tween 20 (Sigma Aldrich, South Africa, catalogue no. P1379) (TBS-Tween or TBST)). Primary antibodies were diluted in 4% ECL-TBST. Primary and secondary antibody dilutions can be found in **Table 2.2**. The membranes were incubated in the respective primary antibody dilutions overnight with shaking at 4°C. Membranes were washed 3 times in 1 X TBST for 5 minutes by shaking and incubated with the secondary antibody (see **Table 2.2**) dilutions in 5% *(w/v)* skim milk powder in 1 X TBST.

The membrane was washed 3 times in 1 X TBST for 5 minutes each time, then placed in 1 X TBS. The membrane was placed in Pierce ECL-chemiluminescent western blotting substrate (ThermoFisher Scientific, USA, catalogue no. 32106). Autoradiography was used to visualize

the protein using Amersham Hyperfilm MP higher performance autoradiography film (AEC Amersham, South Africa, catalogue no. 2890684).

Table 2.2: Antibodies used for Western Blotting

Primary antibody	Primary antibody dilution	Size (kDa)	Secondary antibody	Secondary antibody dilution
GR (Santa Cruz Biotechnology, G-5, sc-393232)	1:3000	95	Anti-mouse HRP conjugate (Santa Cruz Biotechnology, sc-516102)	1:2000
PR A&B (Leica Biosystems, NCL-L-PGR-312)	1:1000	94 & 114	Anti-mouse HRP conjugate (Santa Cruz Biotechnology, sc-2357)	1:3000
AR A&B (Santa Cruz Biotechnology, 441, sc-7305)	1:1000	87 & 110	Anti-mouse HRP conjugate (Santa Cruz Biotechnology, sc-516102)	1:2000
GAPDH (Cell Signalling Technology, 14C10, #2118)	1:500	37	Anti-rabbit HRP conjugate (Santa Cruz Biotechnology, sc-2357)	1:3000

2.10 Statistical analysis

The WHICH clinical data was analyzed in a modified intention to treat (MITT) population, that included data from all 193 women (DMPA-IM n = 99; NET-EN n = 94). In a separate study performed by the Hapgood laboratory, non-study progestins levonorgestrel, nestorone, etonogestrel, and gestodene were quantified in the serum at D0 and 25W of the WHICH cohort, as well as the study progestins MPA and NET. For this thesis, data were also analyzed in a per protocol population (PP) by further excluding participants for whom an above threshold value (1.5 nM) of non-study progestin was detected in their serum at either D0 or 25W (Avenant *et al.*, 2023). Additionally, women in the DMPA-IM arm that had NET concentrations higher than 1.5 nM, at either baseline or 25W, and women in the NET-EN arm that had MPA concentrations higher than 1.5 nM, at either baseline or 25W, were excluded. After the exclusion criteria were met, there were 153 WHICH participants from the MRU and ECRU sites that formed the PP population (DMPA-IM n = 87; NET-EN n = 65). All statistical analysis was done in GraphPad Prism (version 9). A Shapiro-Wilk test was performed to test for normality of the data and indicated that all the in vitro data had a parametric distribution, while the WHICH clinical trial data was non-parametrically distributed. For the WHICH clinical hormonal data, the Wilcoxon matched-pairs signed rank test was used for within group/arm analysis (within the DMPA arm comparing D0 to 25W, or within the NET-EN arm comparing D0 to 25W), while the Mann Whitney test was performed when comparing between groups/arms (DMPA D0 vs NET-EN D0, or DMPA 25W vs NET-EN 25W), as well as for the absolute change (25W – D0) between groups/arms.

For in vitro data, a One-way ANOVA was performed on parametric data, where the responses of all individual ligands were compared to each other. In experiments where the responses by individual ligands and ligands in combination with each other were tested, a Two-way ANOVA was done to compare these two variables in this parametric data. If the One-way ANOVA was significant overall, a post-hoc unpaired one-tailed or two-tailed t-test was performed to detect significant differences (p-value < 0,05) between the ligands, as indicated in the figure legends. An unpaired one-tailed t-test was done in the case where a directional hypothesis was used. Therefore, the gene response by a ligand could only increase or decrease from baseline (i.e. vehicle that is set to 1). Conversely, a non-directional hypothesis was used in an unpaired two-tailed t-test. This t-test does not assume a specific direction that one ligand will have on the gene response when in combination with another ligand. Instead, this test measures whether

the combination of two ligands significantly changes the gene response compared to the baseline set by one of the ligands (i.e. neither of the ligands was set to 1 and both had a standard deviation/error).

Chapter 3: E2, LH, FSH and GnRH concentrations in women randomized to DMPA-IM and

NET-EN

3.1 In both DMPA-IM and NET-EN groups, E2 is suppressed

In order to verify that the population of women used in this study produced similar E2 results as reported for the whole WHICH cohort (DMPA-IM n=262; NET-EN n=259), one aim of this thesis was to analyze E2 levels in a MITT subpopulation of the whole WHICH cohort (DMPA-IM n=99; NET-EN n=94) for whom levels of other HPO hormones were available and analyzed in this thesis. In addition, the published WHICH E2 data (Singata-Madliki *et al.,* 2024) did not correct for non-study progestin levels. Therefore, another aim was to determine if non-study progestins could confound the reported E2 levels. This was determined by analyzing a PP population (DMPA-IM n=87; NET-EN n=65) based on excluding non-study progestin levels.

In the DMPA-IM group, the median E2 concentration decreased by 3,3-fold from 257 pmol/L at D0 to 79 pmol/L at 25W in the MITT population (**Figure 3.1 A and Table 3.1**). In the NET-EN group, the median E2 concentration decreased significantly by 2,6-fold from 179,9 pmol/L at D0 to 69,5 pmol/L at 25W in the MITT population (**Figure 3.1 A and Table 3.1**). In the MITT population, the median E2 levels in the NET-EN group at 25W were lower than the median E2 levels in DMPA-IM group at 25W (**Table 3.1**).

In the PP population, the median E2 levels in the DMPA-IM group decreased by 3,3 -fold from 257 pmol/L at D0 to 77 pmol/L at 25W (**Figure 3.1 B and Table 3.1**). The median E2 levels decreased in the NET-EN group by 4,1-fold from 297,5 pmol/L at D0 to 72 pmol/L at 25W in the PP population (**Figure 3.1 B and Table 3.1**). The significant difference in the median E2 levels at 25W between DMPA-IM and NET-EN observed in the MITT population was lost in the PP population (**Table 3.1**). However, there was still a near to significant difference in median E2 levels at 25W between the DMPA-IM and NET-EN groups (p=0,095) (**Table 3.1**).

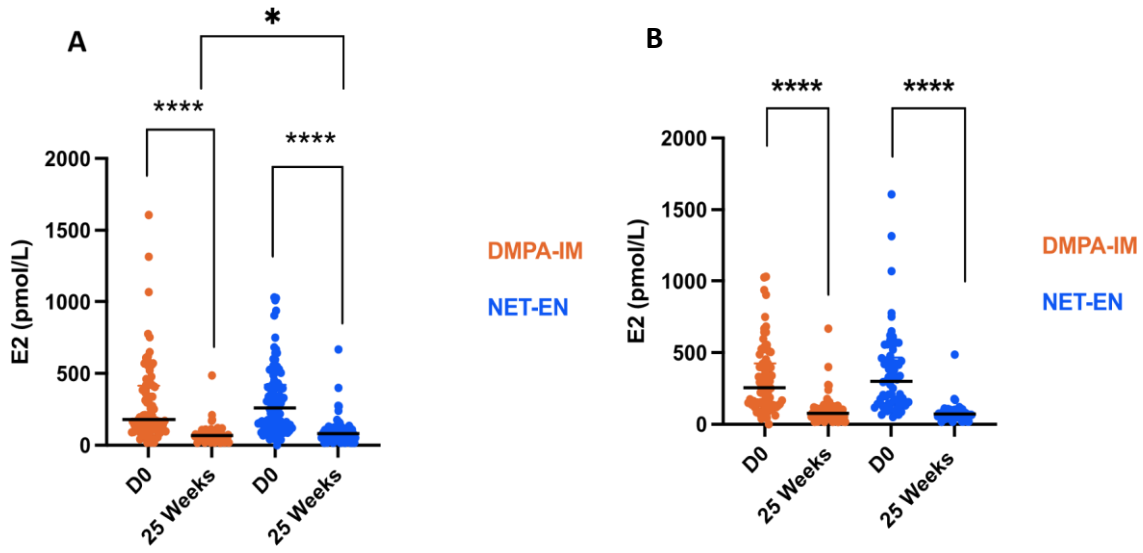


Figure 3.1. E2 levels are suppressed in both DMPA and NET-EN groups. *E2 (pmol/L) in the MITT population (**A**) and PP population (**B**). Graphs indicate median with interquartile range (IQR). In **A,** n=193 (DMPA-IM n=99 and NET-EN n=94) and in **B**, n=172 (DMPA-IM n=87 and NET-EN =65). Within group analysis - Wilcoxon matched-pairs signed rank test; Between groups (D0 vs D0 & 25W vs 25W) - Mann Whitney test. Statistical significance is denoted by * or ****, representing p-values <0,05 or <0,0001, respectively.*

Table 3.1: Summary of E2 serum levels in women randomized to DMPA-IM and NET-EN

	MITT population					PP population				
	DMPA-IM		NET-EN		DMPA-IM vs NET-EN	DMPA-IM		NET-EN		DMPA-IM vs NET-EN
	Median (IQR)	n	Median (IQR)	n	p-value	Median (IQR)	n	Median (IQR)	n	p-value
E2 (pmol/L)										
D0	257 (123423)	99	179,9 (110,8416,3)	94	0,3889	257(132-426)	87	297,5 (148,5-467)	65	0,2926

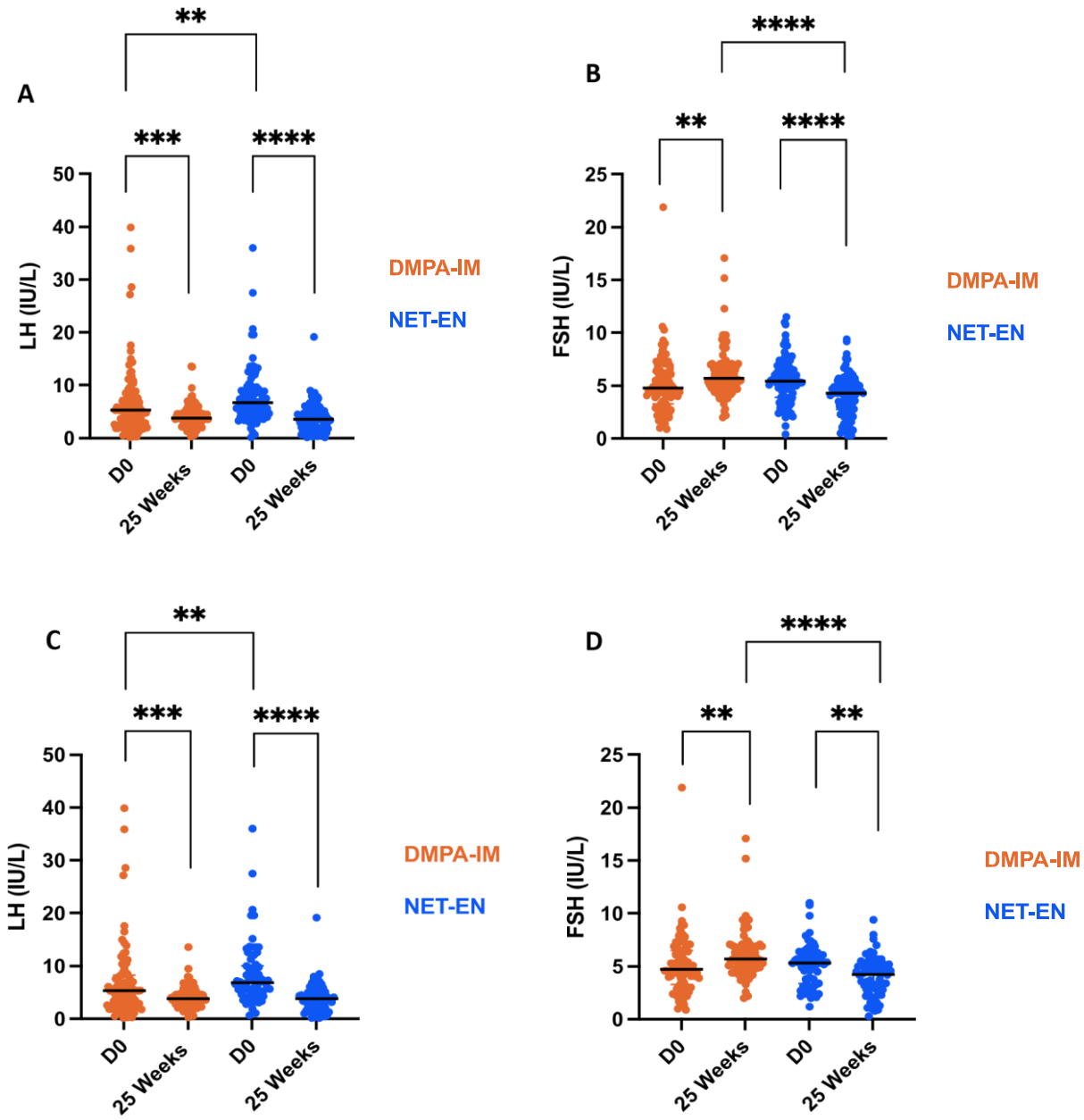
25W	79 (56-108)	99	69,5 (59,85-87,23)	94	**0,0414**	77 (56-109)	87	72 (59,5-88)	65	0,0947
Change (25W-D0)	-147 (-326- 43,2)	99	-96,95 (-345,1- 40,85)	94	0,6802	-147,4 (-326,2 44,8)	87	-211,8 (-402- 67,25)	65	0,1662
Change p-value	**<0,0001**		**<0,0001**			**<0,0001**		**<0,0001**		

**Table represents median with IQR (25th-75th Percentile). Within group analysis – Wilcoxon matched pairs signed rank test. #: Between groups (D0 vs D0 & 25W vs 25W) and Change (absolute 25W – D0) - Mann Whitney test.*

3.2 LH levels decreased in both groups, while FSH levels increased in the DMPA-IM group and decreased in the NET-EN group at 25W

Given that both the DMPA-IM and NET-EN groups exhibited suppressed E2 levels in the MITT and PP populations used in this thesis, the DMPA-IM- and NET-EN-induced regulation of LH and FSH was next investigated.

In the MITT population, the median LH levels decreased by 1,4-fold from 5,3 IU/L at D0 to 3,75 IU/L at 25W in the DMPA-IM group (**Figure 3.2 A and Table 3.2**). In the MITT population, for the NET-EN group, the median LH levels decreased by 2-fold, from 6,7 IU/L at D0 to 3,5 IU/L at 25W (**Figure 3.2 A and Table 3.2**). The DMPA-IM group had significantly lower LH levels at D0 than the NET-EN group (p=0,021) in the MITT population (**Table 3.2**). The decreased change in median LH levels was larger in the NET-EN group compared to the DMPA-IM group in the MITT population (p=0,0051) (**Table 3.2**). The median FSH levels increased by 1,2-fold from 4,8 at D0 to 5,7 IU/L at 25W, in the DMPA-IM group (**Figure 3.2 B and Table 3.2**). Conversely, in the NET-EN group, the median FSH levels decreased by 1,3-fold from 5,40 at D0 to 4,3 IU/L at 25W in the MITT population (**Figure 3.2 B and Table 3.2**). There was no significant difference in the FSH levels between the 2 groups at D0 (**Table 3.2**). However, the DMPA-IM group had higher FSH levels than the NET-EN group at 25W (p<0,0001) (**Table 3.2**).
Similarly, there was a significant difference in the change values between the two groups (p<0,0001) (**Table 3.2**).



<u>Figure 3.2. LH levels decreased in both groups at 25 W, while FSH levels increased in the DMPA-IM group and decreased in the NET-EN group at 25 W.</u> *LH (IU/L) (**A and C**) and FSH (IU/L) (**B and D**). MITT (**A-B** and PP (**C-D**) populations. Graphs show median IQR (25th and 75th Percentile); Within group analysis – Wilcoxon matched-pairs signed rank test; Between groups (D0 vs D0 & 25W vs 25W) – Mann Whitney test. **,***,**** is denoted as p-values of <0,01, <0,001,<0,0001 respectively.*

In the PP population, median LH levels decreased in the DMPA-IM group by 1-4 fold, from 5,3 IU/L at D0 to 3,8 at 25W (**Figure 3.2 C and Table 3.2**). Similarly, in the NET-EN group the median LH levels decreased by 1.8-fold from 6,9 IU/L at D0 to 3,8 IU/L at 25W (**Table 3.2 C and Table 3.2)**. Median LH levels at D0 were higher in the NET-EN group compared to the DMPA-IM group (**Table 3.2**). However, there was no significant difference in median LH levels at 25W between the 2 groups. In the PP population, the change in median LH levels was larger in the NET-EN group compared to the DMPA-IM group (**Table 3.2**). The median FSH levels increased by 1,2-fold from 4,7 IU/L at D0 to 5,7 IU/L at 25W in the DMPA-IM group (**Figure 3.2 D and Table 3.2**). In contrast, the median FSH levels decreased by 1,2-fold from 5,3 IU/L at D0 to 4,25 IU/L at 25W in the NET-EN group (**Figure 3.2 D and Table 3.2**). There was no significant difference between the 2 groups in the median FSH levels at D0. Conversely, median FSH levels were significantly higher in DMPA-IM group compared to the NET-EN group at 25W. Similarly, there was a significant difference between the 2 groups in the change in median FSH levels (**Table 3.2**). Taken together, LH levels decreased from D0 to 25W for both DMPA-IM and NET-EN groups, while differential regulation of FSH was detected between the two groups, with FSH levels decreasing in the NET-EN group but increasing in the DMPA-IM group.

Table 3.2: Summary of LH and FSH serum levels in women randomized to DMPA-IM and NET-EN

	MITT population					**PP population**				
	DMPA-IM		**NET-EN**		**DMPA-IM vs NET-EN**	**DMPA-IM**		**NET-EN**		**DMPA-IM vs NET-EN**
	Median (IQR)	**n**	**Median (IQR)**	**n**	**p-value**	**Median (IQR)**	**n**	**Median (IQR)**	**n**	**p-value**
LH (IU/L)										
D0	5,3 (2,7-7,7)	98	6,7 (4,5-9,2)	94	**0,0021**	5,3 (2,6-8,3)	87	6,9 (4,7-10,1)	66	**0,003**

25W	3,75 (2,9-5,3)	98	3,5 (1,8-5,3)	94	0,226	3,8 (2,8-5,15)	86	3,8 (1,8-5,4)	64	0,4992
Change (25W-D0)	-1,3 (-3,9- 1,1)	98	-2,4 (-6--0,4)	94	**0,0051**	-1,5 (-4,2- 1,2)	86	-2,3 (-6,8- -0,7)	64	0,0187
Change pvalue	**<0,0001**		**0,0003**			**<0,0001**		**0,0003**		
FSH (IU/L)										
D0	4,8 (3,3-6,3)	99	5,4 (3,9-6,5)	94	0,1305	4,7 (3,3-6,5)	87	5,3 (3,4-6,3)	66	0,5388
25W	5,7 (4,8-6,9)	99	4,3 (2,8-5,3)	94	**<0,0001**	5,7 (4,8-6,9)	87	4,25 (2,8-5,2)	66	**<0,0001**
Change (25W-D0)	0,4 (-0,8-2,8)	99	-1,5 (-3,3-0,8)	94	**<0,0001**	0,4 (-1-2,8)	87	-1,1 (-3,2-1,2)	66	**<0,0001**
Change pvalue	**<0,0001**		**<0,0001**			**0,0044**		**0,0025**		

**Table represents median with IQR (25^{th}-75^{th} Percentile). Within group analysis – Wilcoxon matched pairs signed rank test. #: Between groups (D0 vs D0 & 25W vs 25W) and Change (absolute 25W – D0) - Mann Whitney test.*

3.3 Neither DMPA-IM nor NET-EN detectably change GnRH levels in women at 25W compared to baseline

Having shown that DMPA-IM and NET-EN similarly decrease LH levels, while differentially regulating FSH levels, GnRH levels in the MITT and PP populations were next investigated.

In both the MITT and PP populations, no significant difference was detected in the median GnRH levels at D0 and 25W in both groups (**Figure 3.3 and Table 3.3**). Furthermore, in both the MITT and PP populations, no significant differences were detected in GnRH levels between the groups (**Table 3.3**).

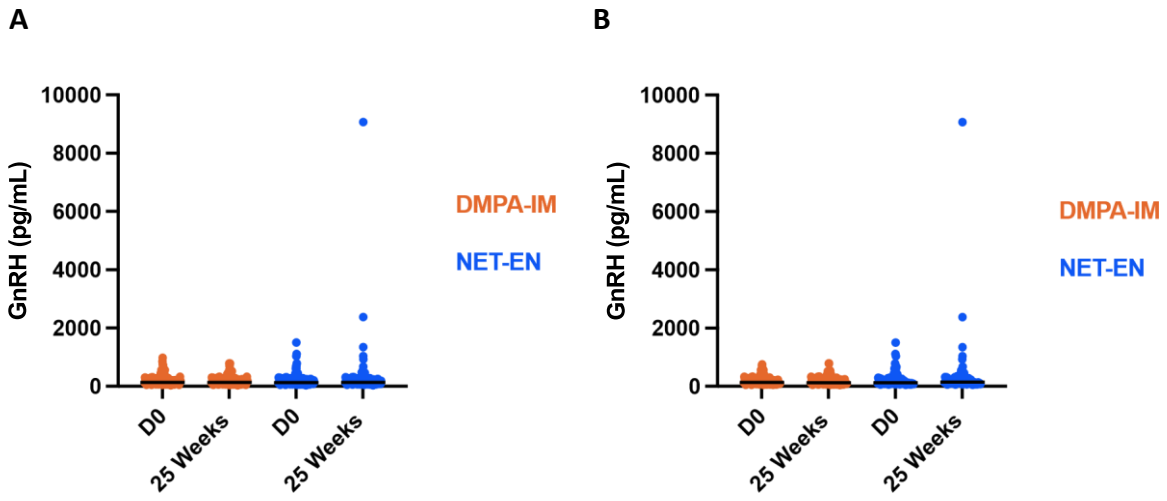


Figure 3.3. Neither DMPA-IM nor NET-EN detectably change GnRH levels in women at 25W compared to D0. *GnRH (pg/ml) in the MITT population (**A**) and PP population (**B**). Graphs indicate median with IQR (25th and 75th Percentile). Within group analysis – Wilcoxon matched-pairs signed rank test, Between groups (D0 vs D0 & 25W vs 25W)– Mann Whitney test.*

Table 3.3: Summary of GnRH serum levels in women randomized to DMPA-IM and NET-EN

	MITT population					PP population				
	DMPA-IM		NET-EN		DMPA-IM vs NET-EN	DMPA-IM		NET-EN		DMPA-IM vs NET-EN
	Median (IQR)	n	Median (IQR)	n	p-value	Median (IQR)	n	Median (IQR)	n	p-value
GnRH (pg/mL)										
D0	139 (88,7-249,4)	99	128,9 (86,69255,8)	93	0,7631	136,6 (88,3 - 248,1)	87	130,5 (87,1273,2)	65	0,7382
25W	135,3 (86,6260,9)	99	136,2 (89,3263,2)	93	0,8251	132,8 (86,6-259)	87	144,5 (98,9303,9)	65	0,1553

Change (25W-D0)	3,66 (-23-18,2)	99	-0,7 (-20,4- 18,5)	93	0,955	3,6 (-20,5-18,2)	87	3,95 (-14,5-29,1)	65	0,4705
Change p-value	0,9591		0,96 l3			0,4563		0,2215		

**Table represents median with IQR (25th-75th Percentile). Within group analysis – Wilcoxon matched pairs signed rank test. $^{\#}$: Between groups (D0 vs D0 & 25W vs 25W) and Change (absolute 25W – D0) - Mann Whitney test.*

Chapter 4: Mechanism of LH and FSH regulation by MPA and NET in a pituitary gonadotrope cell line

The mechanism of MPA- and NET-induced LH and FSH regulation in the pituitary was next investigated given that DMPA-IM and NET-EN reduce E2 and LH levels and differentially regulate FSH levels while, no change from D0 to 25W in GnRH levels were detected

4.1 Regulation of LHβ and FSHβ promoter-reporter activity

4.1.1. MPA and NET alone did not detectably change LHβ promoter-reporter activity.

In order to determine if MPA or NET induce transcription of the LHβ promoter in the LβT2 mouse pituitary gonadotrope cell line, promoter-reporter luciferase assays were conducted. In these experiments, DEX and P4 were included to indicate possible GR- and PR-mediated effects, respectively, on LHβ promoter activity.

There was no significant change caused by MPA, NET, DEX and P4 detected in the LHβ promoter-reporter activity compared to vehicle (**Figure 4.1.1**). These steroid ligands had a mean fold change of 1,07,1,09, 1,03, and 0,703 relative to vehicle, respectively (**Figure 4.1.1**).

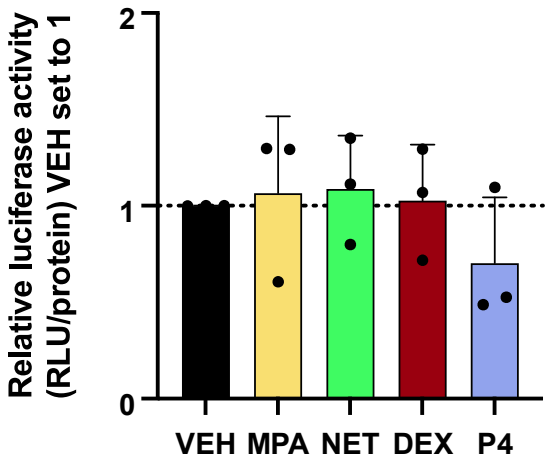


Figure 4.1.1. MPA and NET did not detectably regulate LHβ promoter-reporter activity. *LβT2 cells were grown for 48 hours prior to transfection. The cells were transfected with the -235/5 mLHβ luciferase construct, and after 24 hours, the cells were stimulated with 100 nM MPA, NET, DEX, P4 or 0,1% ethanol (v/v) (vehicle, VEH) for a further 24 hours. Cells were lyzed and luciferase activity was measured and normalized against the protein concentration in each well. The data are plotted as the normalized luciferase activity fold induction relative to vehicle, which was set to 1. A Shapiro-Wilk test found the data to be normally distributed. A One-way ANOVA was performed and was found to be significant (p-value <0.05). A post-hoc unpaired one-tailed t-test was used to determine significant fold induction relative to vehicle. The data shown are from 3 independent experiments. The data are plotted as mean +SD.*

4.1.2 GnRH alone increased LHβ promoter-reporter activity, while MPA and DEX decreased GnRH-induced LHβ promoter-reporter activity, unlike P4.

Given that MPA and NET did not detectably change basal LHβ promoter-reporter activity **(Figure 4.1.1**), this experiment aimed to assess whether MPA and NET modulated GnRH-induced LHβ promoter-reporter activity. In order to achieve this, an LHβ promoter-reporter luciferase assay was performed under the stimulation of the steroid ligands and GnRH alone and with GnRH in combination with the steroid ligands.

Similar to the results in **Figure 4.1.1**, no significant change in basal LHβ promoter activity was detected under the stimulation of MPA, NET or DEX alone (**Figure 4.1.2 A**). P4 alone decreased LHβ promoter activity, by a mean fold change of 0,80 relative to vehicle (p=0,0005) (**Figure 4.1.2 A**). GnRH alone significantly increased LHβ promoter activity by 4,20-fold relative to vehicle (p=0,0007). The steroid ligands in combination with GnRH significantly increased promoter activity, relative to vehicle (**Figure 4.1.2 A)**. However, when promoter activity was measured relative to GnRH, only MPA and DEX significantly decreased GnRH-induced LHβ promoter activity by a mean fold change of 0,70 (p=0,0376) and 0,69 (p=0,0221), respectively (**Figure 4.1.2 B**). In summary, the results showed that MPA and DEX, unlike NET and P4, decreased GnRH-induced LHβ promoter activity.

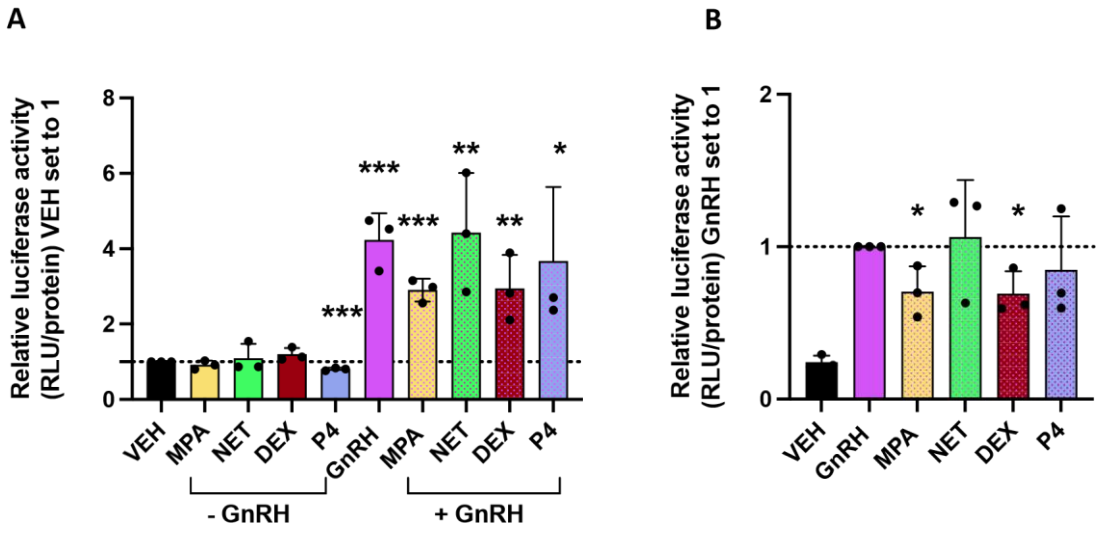


Figure 4.1.2 GnRH alone increased LHβ promoter-reporter activity, while MPA and DEX decreased GnRH-induced LHβ promoter-reporter activity. *LβT2 cells were seeded, transfected and stimulated with 100 nM of the steroid ligands in **Figure 4.1.1** for 18 hours at which time GnRH was added, and the cells were stimulated with both 100 nM of steroid ligands and GnRH for a further 6 hours prior to cell harvesting. Luciferase activity was measured and normalized against protein concentration of each well. The data are plotted as the normalized luciferase activity fold induction relative to either vehicle (VEH) (**A**) or GnRH (**B**), that is set to 1. A Shapiro-Wilk test showed normality of the data. A Two-way (**A**) and One-way (**B**) ANOVA were found to be significant (p-value <0,05). A post-hoc one-tailed and two-tailed unpaired t-test was used to determine significant fold induction relative to vehicle in (**A**) and GnRH in (**B**), respectively. *,**, and ***, denote p-values of <0,05, <0,01,<0,001, respectively. The data shown are from 3 independent experiments (**A and B**). The data was plotted as mean +SD.*

4.1.3 Both MPA and DEX increased FSHβ promoter activity, while no regulation of FSHβ promoter activity by NET and P4 was detected.

In order to explore the regulation of FSHβ promoter-reporter activity by the progestins in the LβT2 cell line, an FSHβ promoter-reporter luciferase assay was conducted.

Both MPA and DEX relative to vehicle significantly upregulated FSHβ promoter-reporter activity by 1,45-fold (p=0,0191) and 2,50-fold (p=0,0007), respectively (**Figure 4.1.3**).

Conversely, there was no significant change detected in the FSHβ promoter activity under the stimulation of NET or P4 (**Figure 4.1.3**).

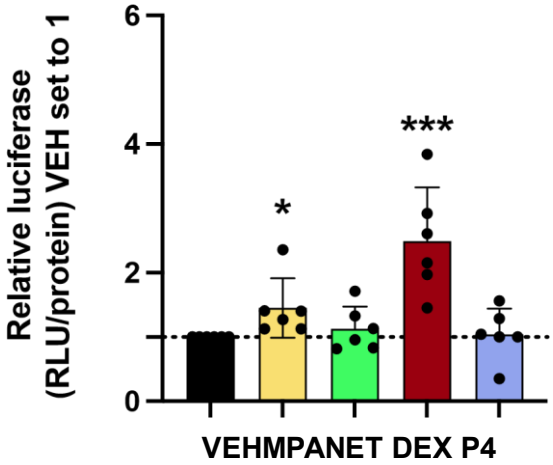


Figure 4.1.3. Both MPA and DEX increased FSHβ promoter activity, while NET and P4 did not detectably regulate FSHβ promoter activity. *LβT2 cells were seeded, transfected with the mFSHβ (1990) construct and stimulated with the indicated ligands as per* ***Figure 4.1.1****. Luciferase activity was measured and normalized against the protein concentration in each well. The data are plotted as the normalized luciferase activity fold induction relative to vehicle, that is set to 1. A Shapiro-Wilk test showed data to be normally distributed. A One-way ANOVA was performed and was found to be significant (p-value <0,05). A post-hoc unpaired one-tailed t-test was used to determine significant fold induction relative to vehicle. *, and ***, is denoted as p-value <0,05, <0,01, <0,001, respectively. The data shown are from 6 independent experiments. The data are plotted as mean +SD*

4.1.4 MPA increased GnRH-induced FSHβ promoter-reporter activity more so than NET.

The aim of this experiment was to determine whether the progestins would regulate GnRH-induced FSHβ promoter activity and whether the addition of GnRH would change the pattern of FSHβ regulation by the steroid ligands that was observed in **Figure 4.1.3**. This was done using a promoter-reporter luciferase assay in LβT2 cells.

MPA and DEX alone significantly upregulated FSHβ promoter activity by 1,26-fold and 2,92fold, respectively, while no regulation by NET and P4 was detected (**Figure 4.1.4 A**). This was similar to the results shown in **Figure 4.1.3**. GnRH upregulated FSHβ promoter activity by 5,88-fold relative to vehicle (p=0,0416) (**Figure 4.1.4 A**). MPA and DEX, in combination with GnRH, increased promoter activity by 28,18-fold (p=0,0014) and 48,81-fold (p=0,0009) relative to vehicle, respectively. Additionally, MPA and GnRH co-stimulation increased promoter activity by 3-fold relative to GnRH alone (p=0,013) (**Figure 4.1.4 B**). DEX in combination with GnRH appeared to have increased promoter activity by 9,17-fold relative to GnRH alone (p-value=0,0669) (**Figure 4.1.4 B**). NET and P4 alone did not detectably change basal promoter activity. However, in combination with GnRH, these steroid ligands significantly upregulated promoter activity by 10,96-fold (p=0,007) and 14-fold (p=0,001) relative to vehicle, respectively (**Figure 4.1.4 A**). A significant increase by NET was detected and increased FSHβ promoter activity by 1,63 (p=0,02), relative to GnRH (**Figure 4.1.4 B**). Compared to GnRH alone, no significant differences were detected with P4 and GnRH co-stimulation. In summary, GnRH increased FSHβ promoter activity. Both MPA and NET increased GnRH-induced FSHβ promoter activity, MPA more so than NET.

A **B**

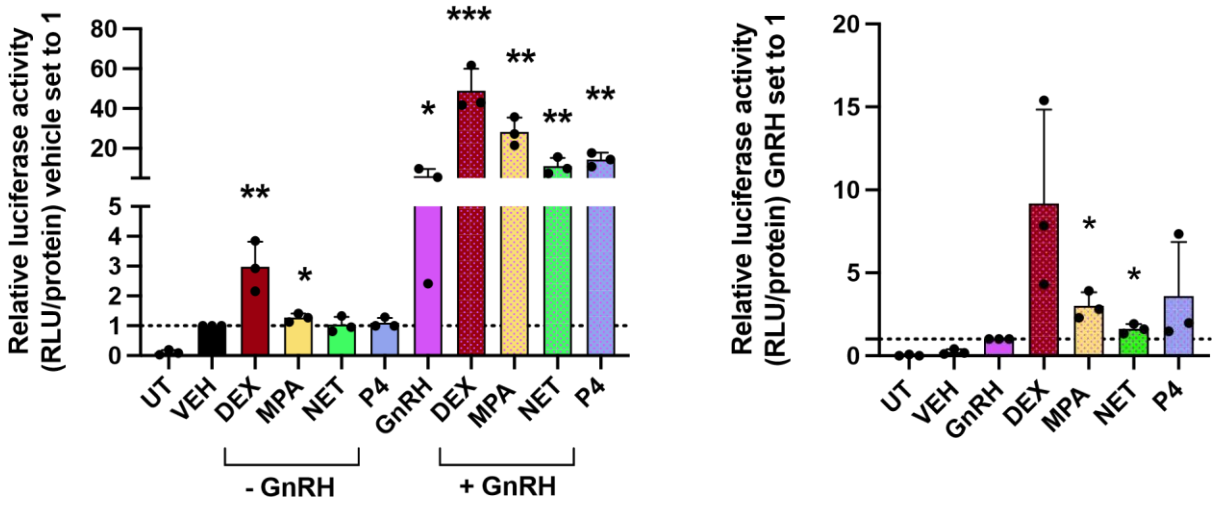


Figure 4.1.4. MPA increased GnRH-induced FSHβ promoter-reporter activity, more so than NET. *LβT2 cells were seeded, transfected and stimulated with 100 nM of the steroid ligands in* ***Figure 4.1.1*** *for 18 hours at which time GnRH was added, and the cells were stimulated with both 100 nM of steroid ligands and GnRH for a further 6 hours, prior to cell harvesting. Luciferase activity was measured and normalized against protein concentration in each well. The data are plotted as the normalized luciferase activity fold induction relative to either vehicle (**A**) or GnRH (**B**), that is set to 1. A Shapiro Wilk test showed normality of data. An ordinary Two-way (**A**) and One-way (**B**) ANOVA was found to be significant (p-value <0,05). A post-hoc one-tailed and two-tailed unpaired t-test was used to determine significant fold induction relative to vehicle (**A**) and GnRH (**B**), respectively. *, **, ***, is denoted as p-value <0,05, <0,01, <0,001, respectively. The data shown are from 3 independent experiments. (**A and B**). The data are plotted as mean +SD.*

4.2 Regulation of LHβ and FSHβ endogenous gene expression

The previous LHβ promoter-reporter results showed transcriptional regulation by MPA and DEX in the presence of GnRH. Similarly, in the FSHβ promoter-reporter assay, there is transcriptional regulation by MPA and DEX both alone and in combination with GnRH. Given these results, it was important to investigate whether these transcriptional effects appear consistent with changes in mRNA levels.

4.2.1 Steroid ligands and GnRH alone and in combination did not detectably regulate LHβ mRNA levels

The aim of this experiment was to test whether the same trend of LHβ promoter activity regulation by the steroid ligands differed or was maintained at a post-transcriptional level. RT-qPCR was performed to measure endogenous LHβ mRNA expression relative to the housekeeping gene, TBP.

Both MPA and NET exerted no detectable significant change in basal LHβ mRNA levels (**Figure 4.2.1.1**). P4 did not regulate LHβ mRNA in the absence of GnRH (**Figure 4.2.1.1**). Surprisingly, GnRH did not upregulate LHβ mRNA levels (**Figure 4.2.1.2**). Furthermore, all the steroid ligands in combination with GnRH did not alter LHβ mRNA levels in comparison to vehicle or GnRH (**Figure 4.2.1.2**). This is in contrast to the results seen at the promoter level **in Figure 4.1.2**.

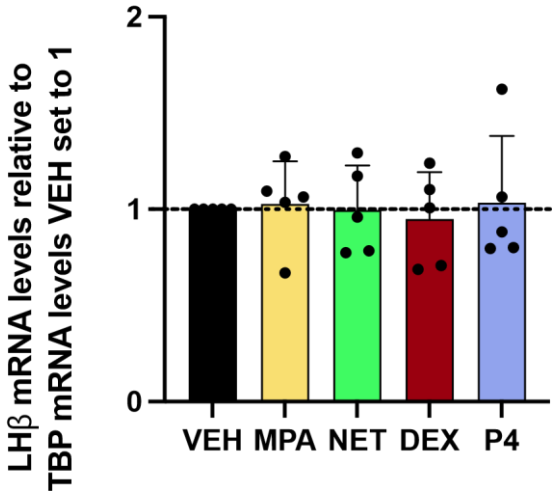


Figure 4.2.1.1. Steroid ligands alone did not regulate LHβ mRNA levels. *LβT2 cells were grown for 24 hours prior to treatment with steroid ligands. The cells were treated with 100 nM of each steroid ligand and ethanol (vehicle, VEH) for 8 hours. Subsequently, RNA was isolated and cDNA was synthesized. RT-qPCR was conducted and the expression of FSH mRNA levels was normalized against TBP mRNA levels. The normalized LHβ mRNA levels of the vehicle control are set to 1. A Shapiro-Wilk test was done to test for normality. A One-way ANOVA was done. All treatment groups in the one-way were found to be non-significant (p-value >0,05). The data shown are from 5 independent experiments. The data are plotted as mean +SD.*

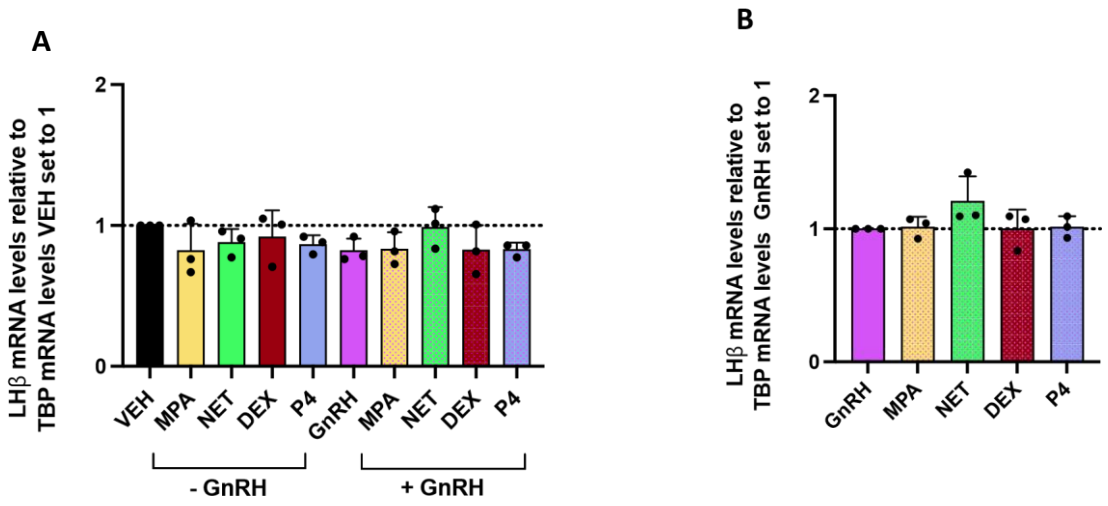


Figure 4.2.1.2. Steroid ligands in combination with GnRH did not regulate LHβ mRNA levels. *LβT2 cells were grown for 24 hours prior to treatment with steroid ligands. The cells were treated with 100 nM of each steroid ligand and ethanol (vehicle, VEH) for 8 hours. 100 nM GnRH was added to the cells alone or to cells already stimulated with another steroid ligand, for the last 6 hours (**A and B**). Subsequently, RNA was isolated and cDNA was synthesized. RT-qPCR was conducted and the expression of LHβ mRNA levels was normalized against TBP mRNA levels. In **A**, the normalized LHβ mRNA levels of the vehicle control are set to 1. In **B**, the normalized LHβ mRNA levels of GnRH are set to 1. A Shapiro-Wilk test was done to test for normality (**A and B**). A One-way ANOVA was done in **B** and a ordinary Two-way ANOVA was done in **A**. All treatment groups in both the One-way and Two-way ANOVA tests were found to be non-significant (p-value >0,05). The data shown are from 3 independent experiments. The data are plotted as mean +SD.*

4.2.2. MPA and DEX, but not NET and P4, upregulated FSHβ mRNA levels

Similar to section 4.2.1 for LHβ, the next aim was to investigate whether the pattern of FSH transcriptional regulation by the progestins, as found in the promoter-reporter assays, was comparable at an mRNA level. In order to investigate this, FSHβ mRNA levels relative to TBP mRNA levels in response to progestins were determined by RT qPCR.

Results with MPA and DEX followed the same trend as those obtained in the FSHβ promoter reporter assays (**Figure 4.1.3)**. MPA increased FSHβ mRNA by 0,30-fold relative to vehicle (p=0,0401) **(Figure 4.2.2**). Similarly, DEX upregulated FSHβ mRNA by 2,83-fold relative to

vehicle (p=0,0044) (**Figure 4.2.2**). In contrast, no significant regulation of FSHβ mRNA levels by NET and P4 was detectable. In summary, the effects of the progestins alone on FSHβ mRNA levels and on FSHβ promoter activity were similar.

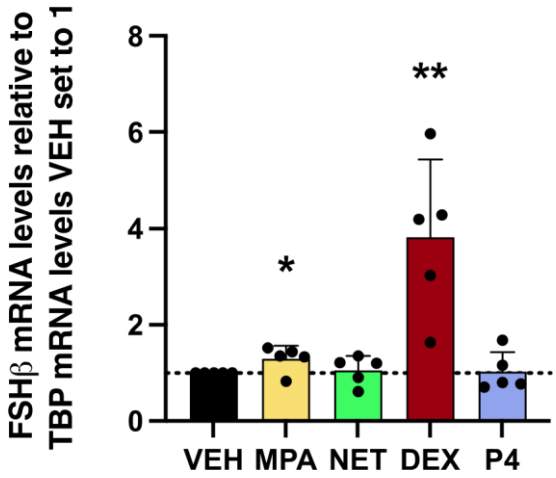


Figure 4.2.2 MPA and DEX, but not NET and P4, upregulated FSHβ mRNA levels. *LβT2 cells were seeded and stimulated by the selected ligands as per Figure 4.2.1A. Subsequently, RNA was isolated, and cDNA was synthesized. RT-qPCR was conducted and the expression of FSHβ mRNA levels was normalized against TBP mRNA levels. A Shapiro-Wilk test was done to test for normality. A One-way ANOVA was found to be significant (p-value <0,05). A post-hoc unpaired one-tailed t-test was used to determine significant fold induction relative to vehicle control. *, **, and***, is denoted as p-value <0,05, <0,01 ,<0,001, respectively. The data shown are from 5 independent experiments. The data are plotted as mean +SD.*

4.2.3. Both MPA and DEX increased GnRH-induced FSHβ expression, unlike NET and P4.

Similarly to **Figure 4.1.4**, this experiment investigated the effects of GnRH alone or in combination with the steroids, on FSHβ mRNA levels.

Similarly to **Figure 4.2.2**, MPA increased FSHβ mRNA by a mean fold change of 2 (p=0,0176) and 5,1 (p=0,0015), respectively (**Figure 4.2.3 A**). Additionally, GnRH alone increased FSHβ mRNA by a mean fold change of 2,6 (p=0,0043) (**Figure 4.2.3 A**). No significant regulation of FSHβ mRNA was detected with NET or P4 alone (**Figure 4.2.3 A**). Only MPA and DEX

upregulated GnRH-induced FSHβ mRNA expression, with a mean fold change of 1,6 (p=0,025) and 3,5 (p=0,0019) relative to GnRH, respectively (**Figure 4.2.3 B**). P4 resulted in a near-to significant up-regulation of GnRH-induced FSHβ mRNA expression (**Figure 4.2.3 B**), with a mean fold change of 1.3 (p=0,0757), relative to GnRH (**Figure 4.2.3 B**). Therefore, the results were similar to the regulation of FSHβ promoter activity by the steroid ligands, showing that DEX and MPA increased GnRH-induced FSHβ mRNA levels.

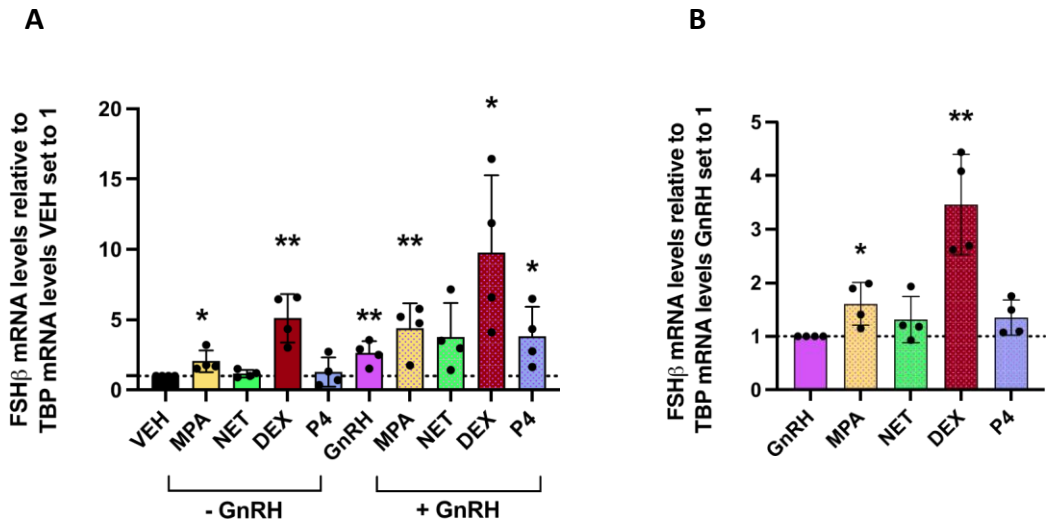


Figure 4.2.3. Both MPA and DEX increased GnRH-induced FSHβ mRNA levels, unlike NET and P4. *LβT2 cells were seeded and stimulated by the selected ligands as per Figure 4.2.1.B. Subsequently, RNA was isolated, and cDNA was synthesized. RT-qPCR was conducted and the expression of FSH*β *mRNA levels was normalized against TBP mRNA levels. In **A**, the normalized FSH*β *mRNA levels of vehicle control are set to 1. In **B**, the normalized FSH*β *mRNA levels of GnRH are set to 1. A Shapiro-Wilk test was done to test for normality. An ordinary Two-way (**A**) and One-way (**B**) ANOVA was found to be significant (p-value <0,05). A post-hoc one-tailed and two-tailed unpaired t-test was used to determine significant fold induction relative to vehicle (**A**) and GnRH (**B**), respectively. *, **, is denoted as p-value <0,05, <0,01, respectively. The data shown are from 3 independent experiments (**A and B**). The data are plotted as mean +SD.*

4.2.4. MPA and DEX alone decreased Cga mRNA levels.

The aim of this experiment was to study whether MPA and NET alone or in combination with GnRH would regulate mRNA for α subunit, Cga, that is shared by both LH and FSH. This was done by RT-qPCR in LβT2 cells.

In **Figure 4.2.4,** only MPA and DEX reduced Cga mRNA levels by a mean fold change of 0,18 (p=0,0458) and 0,45 (p=0,029) relative to vehicle, respectively **(Figure 4.2.4 A**). NET, P4 and GnRH alone did not have a detectable change in Cga mRNA levels (**Figure 4.2.4 A**). There was no change detected in Cga mRNA levels when the steroid ligands were in combination with GnRH (**Figure 4.2.4 A**). However, there appeared to be a trend whereby DEX and P4 suppressed the non-significant induction by GnRH (**Figure 4.2.4 B**). In summary, only MPA and DEX alone significantly decreased Cga mRNA levels.

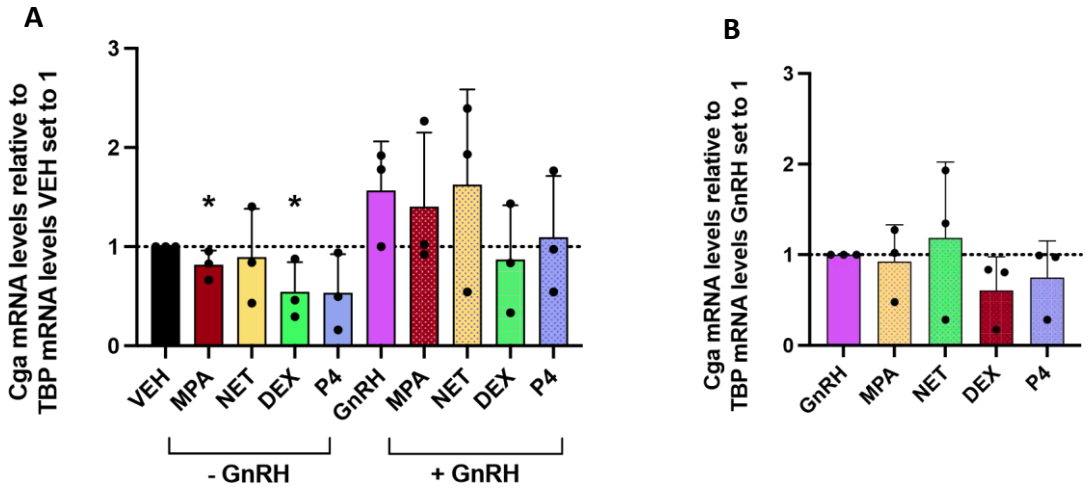


Figure 4.2.4. MPA and DEX decreased Cga mRNA levels. *LβT2 cells were seeded and stimulated by the selected ligands as per Figure 4.2.1.B. Following the stimulation, RNA was harvested, and cDNA was synthesized. A RT-qPCR was performed to measure Cga mRNA levels and normalized against TBP mRNA levels. In **A**, the normalized Cga mRNA levels of vehicle control are set to 1. In **B,** the normalized Cga mRNA levels of GnRH are set to. A Shapiro-Wilk test was done to test for normality. A Two-way ANOVA was done and was found to be significant (p-value <0,05) in **A**. However, no significance was found in a One-way ANOVA done in **B.** A post-hoc unpaired one-tailed t-test was performed to determine significant fold induction relative to vehicle. *, is denoted as p-value <0,05.*

The data shown are from 3 independent experiments. The data are plotted as mean +SD.

4.3 Regulation of LH and FSH secretion

FSH protein secretion was investigated; however, the E-Elabscience ELISA kit used may not have been sensitive enough to be able to detect low levels of FSH secreted from the LβT2 cells, or the LβT2 cells may not have secreted FSH under the stimulation with the selected hormone ligands (**Addendum C, Figure S2**). The average absorbance for LβT2 cell supernatants after vehicle treatment (background) was below the absorbance measured from the LβT2 cell supernatants after treatment with ligands. Therefore, after the subtraction of the background absorbance of the solvent dilutent (without cell supernatant) from the measured absorbance after ligand stimulation, only negative values were obtained (**Addendum C, Figure S2**).

4.3.1 GnRH alone increased LH peptide secretion, while steroid ligands did not change GnRH-induced LH secretion.

The previous results showed that both MPA and DEX in the presence of GnRH, decreased LHβ promoter activity, but none of the steroid ligands regulated LHβ mRNA levels, implying that post-transcriptional regulation could have modulated the transcriptional effects observed in the LHβreporter assays. In addition, while no change in LHβ mRNA was detected, both MPA and DEX reduced Cga mRNA (the α subunit) levels. It was therefore important to determine the regulation of LH protein secretion by the steroid ligands. This would give insight into whether there may be another mechanism of progestin regulation at the level of protein secretion. In order to study this, an LH ELISA was done to measure LH peptide secreted into the medium from the LβT2 cells.

In **Figure 4.3.1 A,** none of the steroid ligands alone changed LH secretion relative to vehicle. GnRH alone increased LH secretion by 2,99-fold (p=0,0032) **(Figure 4.3.1 A)**. Although there was a significant increase in LH secretion by the steroid ligands in combination with GnRH

relative to vehicle, none of the steroid ligands regulated GnRH-induced LH secretion, relative to GnRH alone **(Figure 4.3.1 B).** Therefore, this result shows that the steroid ligands do not regulate basal or GnRH-induced LH secretion under these conditions.

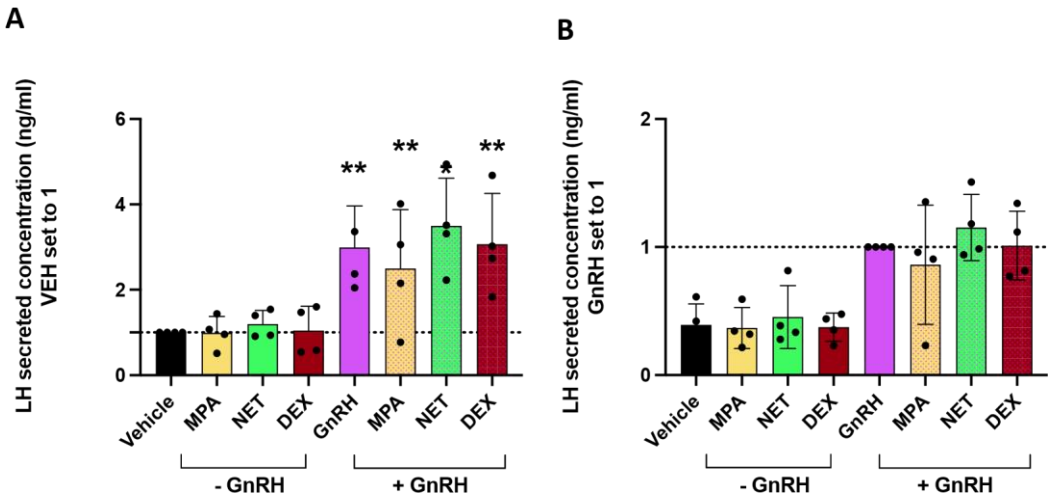


Figure 4.3.1. GnRH, but not steroids alone, increased LH peptide secretion, and steroids did not detectably affect GnRH-induced LH secretion. *LβT2 cells were grown overnight and stimulated with 100 nM of either steroid ligands or 0,1% ethanol and sterile water (v/v) (vehicle) for 8 hours. 100 nM GnRH was added alone and in combination with the other steroid ligands for the final 6 hours. Following the stimulation, supernatant media was collected. A mouse LH ELISA kit (section **2.8**) was used to measure LH secretion as per the manufacturer's instructions. In **A,** the data are represented as fold change relative to the vehicle control with vehicle set as 1. In **B,** the data are represented as fold change relative to GnRH with GnRH set as 1. A Shapiro-Wilk test was done to test for normality of data in both **A and B.** An ordinary two-way (**A**) and one-way (**B**) ANOVA was found to be significant (p-value <0,05). In both **A and B**, a post-hoc un-paired parametric one-tailed and two-tailed t-test was conducted to test for significant fold change relative to vehicle (**A**) and GnRH (**B**), respectively (p-value <0,05). *,**,***, is denoted as p-value <0.05, <0.01, <0.001, respectively. The data shown are from 5 independent experiments (**A and B**). The data are plotted as mean +/±SD.*

4.4 The involvement of the SRs in the regulation of LH and FSH

MPA and NET can mediate their activations via various members of the SR family. MPA can activate the GR, AR, and PR (Pérez-Palacois *et al.*, 1983; Ronacher *et al.*, 2009; Stanczyk *et al.*, 2013) while NET activates the PR and AR (Stanczyk *et al.*, 2013; Africander, Storbeck and Hapgood, 2014). The use of DEX and P4 (a GR- and PR-specific agonist, respectively) throughout this thesis provides insight into the involvement of the GR, and not the PR, in mediating the responses observed in LH and FSH regulation. This is because MPA and DEX have shared a similar pattern in the regulation of LH and FSH. Nevertheless, the expression of the respective SRs was next investigated.

4.4.1. GR protein was detected in LβT2 cells

RT-qPCR was done using SR isoform-specific primers to detect endogenous SR mRNA expression in LβT2 cells. GRα, PR-A and PR-B mRNA were detected in the LβT2 cells (**Figure 4.4.1**). The presence of AR mRNA was not investigated.

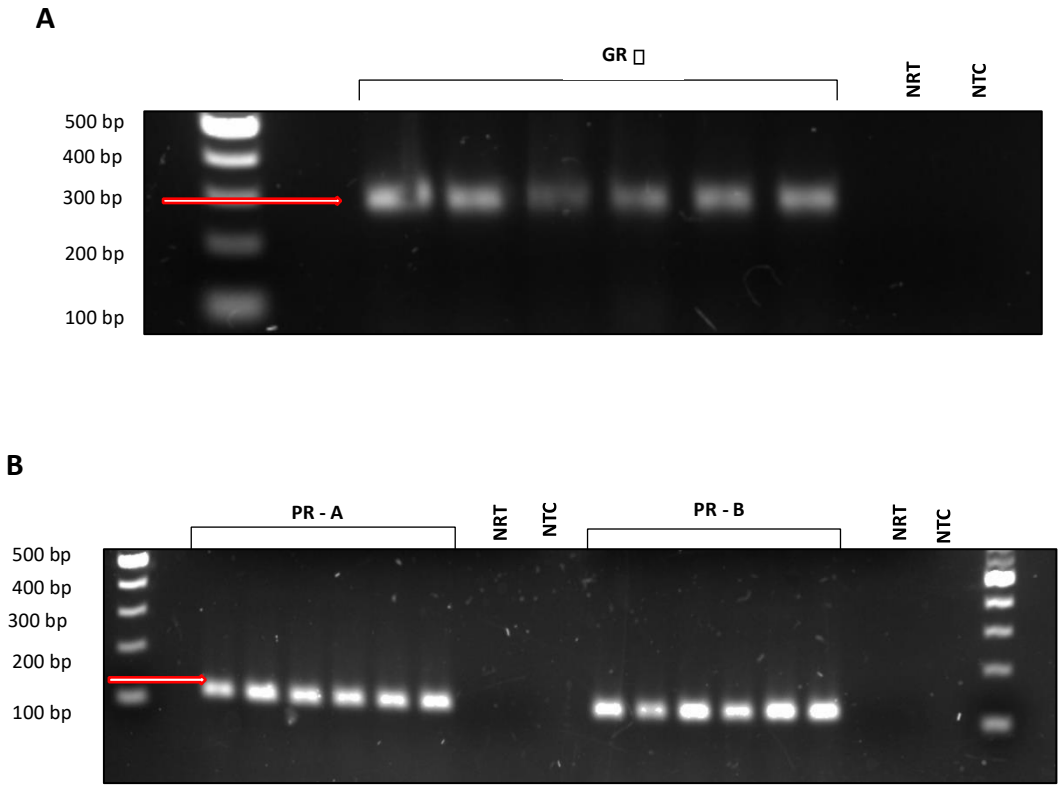


Figure 4.4.1.1 Endogenous GR α, PR-A and PR-B mRNAs were detected in LβT2 cells. *LβT2 cells were seeded at 3 different densities (450 000, 600 000, and 1 000 000) in duplicate and grown for 24 hours. Subsequently, RNA was harvested, and cDNA was synthesized. RT-qPCR was performed on cDNA using primers specific for GR (**A**), PR-A and PR-B (**B**). Thereafter, qPCR products were separated on a 2% agarose gel via electrophoresis, and ethidium bromide was used to visualize separated qPCR products. The red arrows indicate the size of each qPCR product: GR 299 bp (**A**); PR-A and PR-B 120 bp (**B**). NTC = no template control; NRT = no reverse transcriptase control.*

Having shown that LβT2 cells as well express GR and PR-A and PR-B mRNA, a western blot was performed in order to detect these SR proteins as well as the AR-A and AR-B in LβT2 cells **(Figure 4.4.1.2).** Although mRNA was detected for the GR, PR-A, and PR-B, only GR protein levels were detected in LβT2 cells **(Figure 4.4.1.2 A**). PR-A, PR-B and AR-A and AR-B proteins were not detected in LβT2 cells **(Figure 4.4.1.2 B and C).**

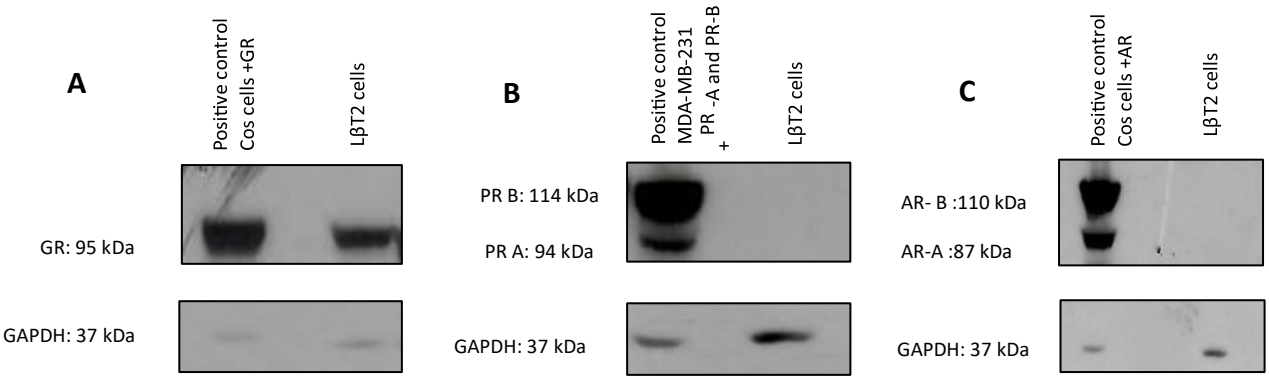


Figure 4.4.1.2 The GR, but not PR or AR protein, is detectable in LβT2 cells. *LβT2 cells were seeded in a 6-well plate at a cell density of 600 000, cells per well for 24 hours. Protein was harvested from the cells using a sample application buffer. Cell lysates were used to perform a western blot using antibodies specific for the GR (**A**), PR-A and PR-B (**B**), AR-A and AR-B (**C**), and GAPDH. Representative blots are shown. The exposure time was 30 seconds.*

4.4.2 The GR is involved in the regulation of GnRH-induced FSHβ promoter activity by MPA and GnRH-induced FSHβ mRNA levels by MPA and DEX.

There was a differential regulation of both the GnRH-induced FSHβ promoter activity and mRNA levels by the progestins. Only the GR protein was detectable in LβT2 cells by western blot (**Figure 4.4.1.2**). Therefore, the next aim was to investigate whether the GR was involved in the effects of progestins on the GnRH-induced increase of FSHβ promoter activity and gene expression. In order to determine this, cells were co-stimulated with select steroid ligands in the absence and presence of RU486, a GR/PR antagonist, and FSHβ promoter-reporter activity and mRNA levels were measured in the presence of GnRH. The GR involvement in the regulation of the LHβ promoter-reporter activity was also investigated (**Addendum C, Figure S5**). However, due to time constraints and inconsistencies in the results, this section focused on the regulation of FSHβ promoter-reporter activity and mRNA levels.

In the absence of RU486, MPA significantly increased GnRH-induced FSHβ promoter activity by 4,86-fold (p=0,0366), while no significant difference was detected with DEX **(Figure 4.4.3 A),** similar to the results shown in **Figure 4.1.4 A**. Both MPA and DEX increased GnRH-induced FSHβ mRNA levels by 1,77-fold (p=0,0479) and 2,94-fold (p=0,0211), respectively, in the absence of RU486 **(Figure 4.4.3 B)**. A change by NET or P4 was not detected in either the GnRH-induced FSHβ promoter activity or mRNA levels in the absence of RU486 **(Figure 4.4.3 A and B)**. Interestingly, in the presence of RU486, the significant increase in GnRH-induced FSH promoter activity and mRNA levels by MPA was lost. Similarly, the significant increase in GnRH-induced FSHβ mRNA levels by DEX was lost in the presence of RU486. In short, the differential regulation of GnRH-induced FSHβ expression by MPA and NET was not detected in the presence of a GR antagonist.

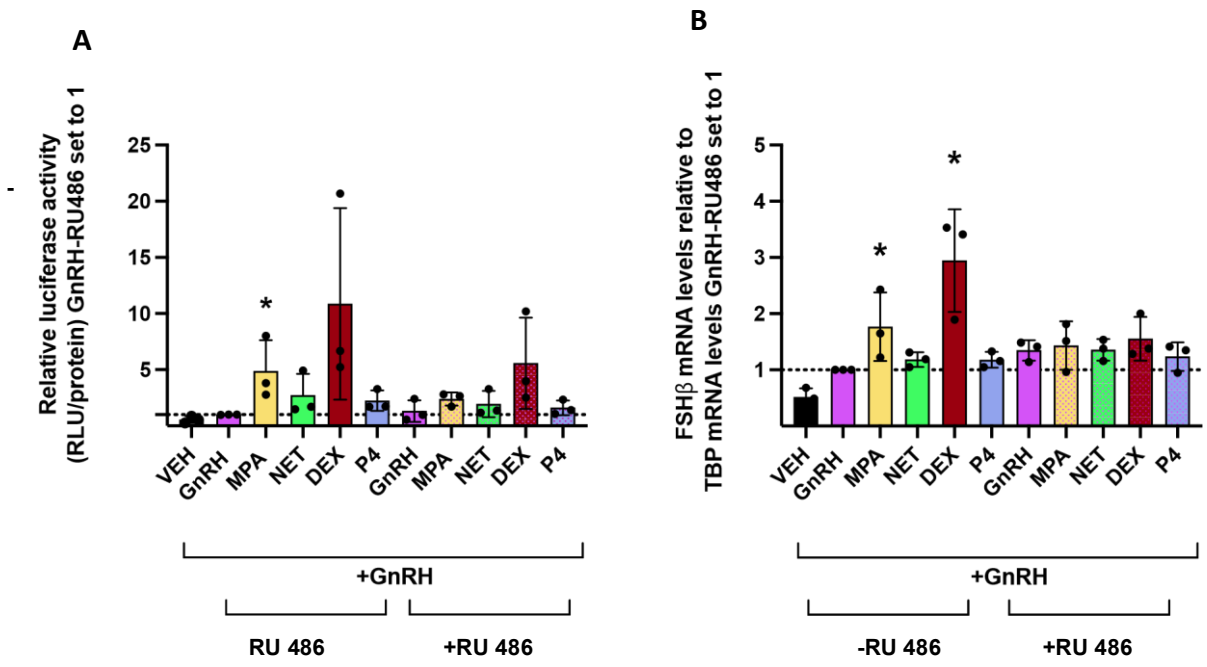


Figure 4.4.2. GR may be involved in the regulation of FSHβ promoter activity by DEX and MPA in combination with GnRH. *In **A,** LβT2 cells were seeded, transfected, and stimulated with the selected ligands as per Figure 4.1.3.B. Cells were co-stimulated with 100 nM RU486 for 24 hours in combination with the ligands mentioned in Figure 4.1.3.B. Luciferase activity was measured and normalized against the protein concentration in each well. The data are plotted as the normalized luciferase activity fold induction relative to GnRH in the absence of RU486, which is set to 1. In **B,** LβT2 cells were seeded and stimulated with the selected ligands as per Figure 4.2.1.B. Cells were co-stimulated with 100 nM RU486 for 24 hours in combination with the ligands mentioned in Figure 4.2.1.B. RNA was harvested and cDNA was synthesized. A RT-qPCR was performed to measure FSH mRNA levels and normalized against TBP mRNA levels. The normalized FSHβ mRNA levels of GnRH in the absence of RU486 are set to 1. In both **A and B**, a Shapiro-Wilk test showed the data to be normally distributed. A two-way ANOVA was performed and found to be significant (p-value <0,05). A post-hoc unpaired two-tailed t-test was used to determine significant fold induction relative to GnRH in the absence of RU486. *, is denoted as p-value <0,05. The data shown are from 3 independent experiments. The data are plotted as mean ±SD.*

Chapter 5: Discussion

Injectable progestin-only contraceptives are important for reproduction regulation in SSA women (Ayuk *et al.*, 2022). However, the hypoestrogenism caused by these contraceptives is a large concern for this demographic (Hickey, Marino and Tachedjian, 2016; Hapgood, Kaushic and Hel, 2018; Singata-Maliki *et al.,* 2024). E2 is a key hormone that regulates a variety of reproductive functions in women, including the FGT (Molander *et al.*, 1990; Cotreau *et al.*, 2007; Hummelen *et al.*, 2011; Hapgood, Kaushic and Hel, 2018). E2 is also protective against viral infections, more specifically HIV-1 (Smith, Baskin and Marx, 2000; Hapgood, Kaushic and Hel, 2018). Given that DMPA-IM and NET-EN are widely used in the same demographic as women disproportionally infected with HIV-1 in South Africa, it is important to investigate how hypoestrogenism is caused by these contraceptives (Ayuk *et al.*, 2022; UNAIDS, 2022). Literature has established that the pituitary hormones LH and FSH are critical regulators of E2 synthesis (Raju *et al.*, 2013). Additionally, GnRH contributes to E2 synthesis, given that it is a key overall regulator of LH and FSH (Raju *et al.*, 2013; Atwood and Vadakkadath Meethal, 2016). Given these facts, it is likely that the regulation of GnRH, LH and FSH levels by DMPA-IM and NET-EN contributes to hypoestrogenism in women on these contraceptives. However, there are limited robust studies that compare the relative effects of DMPA-IM and NET-EN on LH and FSH in women. Furthermore, there are no studies that measure GnRH levels in women on contraceptives. Lastly, there are no studies that investigate the regulation of transcription or secretion of the gonadotropins by any contraceptive, at the pituitary level.

This study has addressed these key gaps in the literature in order to understand the regulation of LH and FSH serum levels by DMPA-IM and NET-EN. Therefore, this contributes to further understanding the possible mechanisms behind hypoestrogenism in women on these contraceptives. The WHICH trial provided robust data on the comparative hypoestrogenic effects in women randomized to DMPA-IM (n=262) and NET-EN (n=259) (Singata-Madliki *et al.*, 2024). A secondary study on the WHICH cohort objectively showed that the 25W samples in the WHICH trial were taken at peak serum MPA and NET concentrations and provided evidence on non-study progestin use (Avenant *et al.*, 2023). This thesis is therefore the first study to provide robust clinical data on LH, FSH, and GnRH in a subpopulation of the WHICH cohort of women randomized to DMPA-IM (n=99) and NET-EN (n=94), at peak serum MPA and NET concentrations, and taking non-study progestins into account. Furthermore, the

comparable effects of DMPA-IM and NET-EN on E2 in the same subpopulation of women were analyzed in this thesis. This study is also the first to investigate the mechanisms behind the transcriptional and post-transcriptional regulation of both subunits of LH and FSH, as well as the regulation of secretion of LH and FSH protein by MPA and NET at the pituitary level in a mouse gonadotroph cell line. Additionally, this study was able to expand on the role of the GR in mediating the effects of MPA on FSHβ expression.

5.1 E2, LH, FSH and GnRH levels in women on DMPA-IM and NET-EN.

For the MITT population in this study, median E2 serum concentrations in the DMPA-IM group (D0 257 pmol/L; 25W 79 pmol/L) and NET-EN group (D0 287 pmol/L; 25W 59.9 pmol/L) (**Table 3.1**) were comparable to those reported for the whole WHICH cohort (DMPA-IM: D0 189.4 pmol/L, 25W 76.5 pmol/L; NET-EN: D0 183.2 pmol/L, 25W 69.8 pmol/L) (Singata-Madliki *et al*., 2024). In the DMPA-IM group, the median 25W E2 data from the MITT population indicated levels that were similar to those found in post-menopausal women (7.34 - 158 pmol/L) (**Addendum A Table A2**) and are in agreement with previous studies reporting hypoestrogenic effects in DMPA-IM users (Miller *et al.*, 2000; Clark *et al.*, 2001; Hickey, Marino and Tachedjian, 2016). Interestingly, in the MITT population analyzed in this thesis, NET-EN suppressed E2 levels more than DMPA-IM (**Figure 3.1 A and Table 3.1**). This hypoestrogenic effect in NET-EN users is consistent with some studies (Lawrie *et al.*, 1998; Dabee *et al.*, 2019). However, this result is inconsistent with most other previous studies in NET-EN users, which reported E2 levels were suppressed to early follicular phase levels (Goebelsmann *et al.*, 1979; Landgren and Diczfalusy, 1980; Saleh *et al.*, 1983) (**Addendum A Table A2**). While NET-EN decreased E2 levels more so than DMPA-IM in the MITT population of this study, no significant difference between the two groups was found in the PP population after excluding for non-study progestins (**Figure 3.1 B and Table 3.1**). These results in the PP population are comparable to those reported in the whole WHICH cohort (Singata-Madliki *et al*., 2024). Taken together, the results presented in this thesis showed that both DMPA-IM and NET-EN use, similarly result in hypoestrogenism at postmenopausal levels at peak serum progestin concentrations. This is consistent with the published results for the whole WHICH cohort.

Additionally, the results in this thesis show for the first time that this finding holds after excluding non-study progestins in the study groups.

In the MITT population in this study, median LH serum levels at 25W in both arms (DMPA-IM 4.2 IU/L; NET-EN 3.5 IU/L) were lower compared to the levels reported in the majority of previous studies (6.3 IU/L – 20.0. IU/L), but similar to some others (1.8 IU/L – 5.5 IU/L) (**Addendum A Table 1 and Table 3.2**). The median LH levels at D0 in both arms (DMPA-IM 5.3 IU/L; NET-EN 6.7 IU/L) were comparable to early follicular phase levels in pre-menopausal women (3.7 IU/L -10.4 IU/L) (**Addendum A Table 2**). A decrease in median FSH serum levels at 25W (4.3 IU/L) in women on NET-EN is consistent with previous literature (3.9 IU/L-10 IU/L) (**Table 3.2 and Addendum A Table 1**). Although FSH serum levels at 25W in the DMPA-IM group were similar to the range of FSH levels reported in women on DMPA-IM in the majority of previous research (3.4 IU/L-18.15 IU/L) (**Addendum A Table 1**), FSH serum levels in the DMPA-IM group increased from D0. This is in contrast to the majority of previous research, where FSH levels are unchanged or decreased in DMPA-IM users (Perez-Lopez, L'Hermite and Robyn, 1975; Jeppsson and Johansson, 1976; Jeppsson *et al.*, 1977; Toppozada, Parmar and Fotherby, 1978; Jeppsson *et al.*, 1982) (**Addendum A Table 1**). The median serum FSH levels at D0 for both study groups (DMPA-IM 4.8 IU/L; NET-EN 5.4 IU/L) were similar to FSH levels in either the early follicular phase or the luteal phase in normal-cycling women (**Addendum A Table 2**). The LH:FSH ratio at D0 (DMPA-IM 1,0; NET-EN 1,3) is comparable to the midfollicular phase LH:FSH ratio (1,0-1,6) found in normal-cycling women (**Table 3.2**) (Futterweit, 1984). However, this ratio at 25W (DMPA-IM 0,66; NET-EN 0,77) was significantly decreased from D0 and lower than the LH:FSH ratio during the follicular phase (**Table 3.2**). Furthermore, the LH:FSH ratio was significantly lower in the DMPA-IM group compared to the NET-EN group, due to the higher FSH levels in the DMPA-IM group (**Table 3.2**).

In the PP population in this study, there was still a significant decrease in the median LH serum levels by DMPA-IM and NET-EN at 25W (DMPA-IM 3.8 IU/L; NET-EN 3.8 IU/L), when non-study progestins had been excluded (**Table 3.2**). Similarly, the significant effects on FSH serum levels by DMPA-IM and NET-EN at 25W (DMPA-IM 5.7 IU/L; NET-EN 4.25 IU/L) were not lost in the PP population (**Table 3.2**). There was still a significant decrease in the LH:FSH ratio from D0 (DMPA-IM 0,96; NET-EN 1,51) to 25W (DMPA 0,66 ; NET-EN 0,81) in both groups.

Furthermore, the LH:FSH ratio in the DMPA-IM group was still significantly lower than in the NET-EN group at 25W. These findings are the first to investigate whether non-study progestins may confound the results obtained for the effects of DMPA-IM and NET-EN on LH and FSH levels. Given the fact that no significant effects were lost in the PP population as compared to the MITT population, these results further validate the effects of injectable progestin contraceptives on LH and FSH levels.

No difference in median GnRH levels from 0 to 25W was detected in either contraceptive group (**Figure 3.4 and Table 3.4**). The serum GnRH levels in both groups (DMPA-IM D0 139 pg/mL, 25W 135,3 pg/mL; NET-EN D0 128,9 pg/mL, 25W 136,2 pg/mL) were much higher than the levels previously reported in pre-menopausal women not on hormonal contraceptives (16.5 pg/mL -19.8 pg/mL) (**Addendum A Table 3**). It is important to note that, to the best of the candidate's knowledge, only one study, involving five women, has previously reported GnRH levels in premenopausal women not on contraception (Sarda, Barnes and Nair, 1981), while no one has reported GnRH levels in DMPA-IM and NET-EN users. Thus, these findings serve as robust data for future reference.

The majority of previous studies are likely confounded by multiple factors compared to the WHICH study, which could explain the differences in results. These confounding factors include BMI, ethnicity, injection frequency, sampling time, and statistical methods. In previous studies, few involved more than one injection in women, and LH and FSH levels were not measured in any at the peak serum progestin concentrations (Goldzieher *et al.*, 1970; Perez-Lopez, L'Hermite and Robyn, 1975; Jeppsson and Johansson, 1976; Toppozada, Parmar and Fotherby, 1978; Jeppsson *et al.*, 1982; Siregar, Rita and Yusrawati, 2019; Casteel and Singh, 2022). These factors are likely to influence endogenous HPO hormone levels, such as a stimulatory effect of DMPA-IM on FSH levels (Mishell *et al.*, 1968, 1972; Ortiz *et al.*, 1977; Petta *et al.*, 2001).

5.2 Decreased LH levels by both MPA and NET acting at the pituitary are likely the cause of hypoestrogenism in DMPA-IM and NET-EN users.

The decrease in LH levels by DMPA-IM and NET-EN would likely result in decreased synthesis of endogenous steroids in the ovaries, regulated by LH (Raju *et al.*, 2013). The activation of PKA by LH would likely be decreased (Millier, Whitelaw and Smyth, 1994; Miller *et al.,* 2011; Raju *et al.*, 2013), which would likely decrease the expression of transcription factors and steroidogenic enzymes, specifically 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD, that are needed to synthesize androgens in the theca cells (Millier, Whitelaw and Smyth, 1994; Miller *et al.,* 2011; Raju *et al.*, 2013). A decrease in androgen levels would likely decrease the final E2 levels due to less T substrate (Gervásio *et al.*, 2014). This is consistent with reports linking low LH levels (less than 10 IU/L) (Gordon, 2010) to androgen deficiency (T levels less than 0,87 nmol/L) (Guay and Davis, 2002), which impairs follicular development (Vendola *et al.*, 1999; Gervásio *et al.*, 2014) and reports linking folliculogeneis to E2 synthesis (Reed and Carr, 2000; Raju *et al.*, 2013).

While this thesis did not investigate T levels, another WHICH secondary study showed that in the whole WHICH cohort, both DMPA-IM and NET-EN reduced T levels in women (Avenant *et al*., 2024). Interestingly, NET-EN reduced T levels significantly more than DMPA-IM (**Addendum A Table 4**) (Avenant *et al*., 2024). This is consistent with the E2 results in the MITT population of this study, whereby NET-EN reduced E2 significantly more than DMPA-IM (**Figure 3.1 A and Table 3.1**). However, this significance was lost in the PP population, although a near-significant decrease was observed (**Figure 3.1 B and Table 3.1**). The PP population excluded non-study progestins at D0 and 25W. Given the loss of significance for E2 levels between the contraceptive groups from the MITT population to the PP population, this indicates that the concentration of an incorrect progestin most likely confounded the result. However, this could also have been due to a loss of power due to fewer women in the PP than in the MITT study. Additionally, the primary WHICH E2 results (from the larger cohort) did not find a significant difference between the E2 suppression in the DMPA-IM group and NET-EN group. Thus, taken together, it is likely that there is no greater hypoestrogenic effect in NET-EN users compared to DMPA-IM users. Regardless of this conclusion, the decrease in T levels in both contraceptive groups from the larger WHICH cohort (Avenant *et al*., 2024), coupled with the decreased LH

levels shown in this study (**Addendum A Table A4**) further imply that these contraceptives may inhibit androgen synthesis to cause their hypoestrogenic effect.

The physiological significance of the differential effect on FSH levels by contraceptives is challenging to understand (**Figure 3.1 and Table 3.1**). It is important to note that the findings of this thesis, when combined with previous research, show that the primary cause of hypoestrogenism in the DMPA-IM and NET-EN groups is likely decreased LH levels, which likely reduces T (or other androgens upstream T production) and hence suppresses E2 levels. FSH reportedly increases the expression of CYP19, which is the steroidogenic enzyme required for the conversion of T to E2 (Millier, Whitelaw and Smyth, 1994; Sahmi, Nicola and Price, 2006). Since T is suppressed in both the DMPA-IM and NET-EN groups, the difference in FSH levels among the contraceptive users may result in relatively less T being converted to E2 in the NET-EN group compared to the DMPA-IM group, due to the effects of FSH on CYP19. Thus, a greater suppression of E2 would be likely in the NET-EN group. However, as discussed in the previous section, a greater hypoestrogenic effect was not detected in the NET-EN group compared to the DMPA-IM group. Therefore, these differential FSH levels between contraceptive groups may not be relevant to hypoestrogenic effects caused by DMPA-IM and NET-EN. While it is difficult to interpret the significance of differential FSH levels in DMPA-IM and NET-EN users in terms of hypoestrogenism, they may have other physiological effects in women.

The LH:FSH ratio at 25W is significantly decreased in the DMPA-IM group compared to the NET-EN group (**Table 3.2**). This is due to increased FSH levels in the DMPA-IM group compared to the decreased FSH levels in the NET-EN group and decreased LH levels in both groups at 25W (**Figure 3.2 and Table 3.2**). A LH:FSH ratio lower than 1 (which is seen in both groups, but more so in the DMPA-IM group at 25W) (**Figure 3.2**) has been reported to be associated with functional hypothalamic amenorrhea (FHA) (Boegl *et al.*, 2024). Interestingly, in the primary WHICH study, the DMPA-IM group has a higher incidence of amenorrhea compared to the NET-EN group (Singata-Madliki *et al.*, 2024). Therefore, the lower LH:FSH ratio for DMPA-IM than NET-EN users, with both ratios being below 1, may result in the greater decrease of amenorrhea seen in the DMPA-IM group. The long-term effects of amenorrhea are linked with increased risk to cardiovascular health, infertility, and osteoporosis (Shufelt, Torbati and Dutra, 2017). Thus, given these associations with amenorrhea, DMPA-IM users may exhibit more of these side effects compared to NET-EN users.

Taken together, while the differential regulation of FSH is difficult to interpret in terms of hypoestrogenism, it may be associated with differential degrees of amenorrhea between the

contraceptives, due to changes in the LH:FSH ratios. The decrease in LH levels by both DMPA-IM and NET-EN most likely plays a dominant role in hypoestrogenism in both contraceptive users. Furthermore, given that neither DMPA-IM nor NET-EN detectably changed GnRH levels, it is likely that regulation of LH levels occurs, at least in part, at the pituitary level.

5.3 Regulation of LH and FSH transcription, mRNA levels and secretion in the pituitary

Currently, there are no previous studies that have investigated the regulation of both LHβ and FSHβ promoter-reporter activity and mRNA levels by MPA and NET. Additionally, there are no studies that have investigated the effect of MPA and NET on LH protein secretion in LβT2 cells. Therefore, the results presented in this study are novel. At the transcriptional level, MPA increases LHβ promoter activity in the presence of GnRH. The increase of GnRH- induced LHβ promoter activity by DEX is as expected from previous studies (Thackray *et al.*, 2009; Breen *et al.*, 2012). The increase by DEX further supports the effects by MPA (due to the glucocorticoid activity of MPA) (**Figures 4.1.1-4.1.2**). Neither MPA, DEX, nor GnRH regulate LHβ mRNA levels (**Figure 4.2.1**). However, there may still be some regulation of LHβ by MPA at the mRNA level not detected in this study, as previous studies on the decrease of LHβ mRNA by DEX (in the presence of GnRH) would support the possible regulation by MPA. The apparent discrepancy between the results in this study and previous studies may be due to the concentration of GnRH used. DEX decreased GnRH-induced LHβ mRNA at 10 nM in previous research in LβT2 cells (Thackray *et al.*, 2009; Breen *et al.*, 2012). However, in this study, 100 nM of GnRH was used. The effects of NET on LHβ transcription and mRNA levels were not detected in the LβT2 cells. Furthermore, the lack of detection of effects by P4 on LH regulation was unexpected. Previous studies in LβT2 cells found P4 to decrease GnRH-induced LHβ transcription and mRNA levels. These findings could have supported possible regulation of LHβ transcription and mRNA levels by NET and MPA, given that both progestins are progestogenic. The regulation by NET, P4, and also MPA may not have been detected in this study due to low levels or no expression of PR protein, as previous studies overexpressed PRB in LβT2 cells (Thackray *et al.*, 2009).

None of the progestins regulated LH secretion in the presence of GnRH in LβT2 cells (**Figure 4.3.1**). It is important to note that there is a large error amongst the data points in **Figure 4.3.1**, and this may indicate a low statistical power. Therefore, more repeats may have to be done in

order to increase the power to detect the possible changes caused by the progestins. Furthermore, the sensitivity of the ELISA may play a role, as this technique may be more sensitive to detecting larger amounts of LH secreted in blood samples compared to LH secreted in LβT2 cell supernatant samples. Interestingly, DEX did not modulate GnRH-induced LH secretion as seen in previous research (McNeilly *et al.*, 2003; Nicol *et al.*, 2004), which could have supported MPA regulation of LH protein secretion. Furthermore, in earlier studies, GnRH-induced LH secretion was modulated by DEX only in the presence of E2 in LβT2 cells (Turgeon *et al.*, 1996). Additionally, DEX targeted the frequency of GnRH pulses in order to have an effect on LH secretion (Turgeon *et al.*, 1996). Lastly, previous studies used radioimmunoassay to measure LH secretion in LβT2 cells, compared to the less sensitive ELISA kit used in this study (Turgeon *et al.*, 1996; McNeilly *et al.*, 2003; Nicol *et al.*, 2004). Therefore, the difference in assay used to detect LH secretion, additional E2 stimulation, and the way GnRH was administered may explain the lack of change in LH secretion by DEX and MPA. Furthermore, the half-life of LH is between 5-30 minutes in rats (Cahoreau, Klett and Combarnous, 2015). However, the half-life of LH has not been investigated in LβT2 cells and may change depending on the oligosaccharide modification (Wide *et al.,* 2009). Thus, given that the secreted LH protein was measured after 8 hours, could also be a factor as to the lack of significant effect by the progestins and DEX. These findings indicate that the regulation of LH in the pituitary by MPA is most likely not occurring predominantly at the transcriptional or mRNA level. This is supported by the DEX results. It is likely that the regulation of LH serum levels by MPA may be at the secretion level, as this would be consistent with previous literature on DEX effects on LH secretion (Turgeon *et al.*, 1996). However, regulation of LH secretion could not be mimicked in the cell line model used in this study. NET likely acts similarly to MPA on the various levels of LH regulation. However, due to the lack of appropriate levels of PR-B protein, these effects could not have been detected.

Similar to LH, the regulation of FSHβ promoter-reporter activity and mRNA levels by MPA compared to NET has not been previously studied. Thus, these results are the first to show the effects of MPA and NET on FSHβ promoter-reporter activity and mRNA levels. MPA increased basal and GnRH-induced FSHβ transcription and mRNA levels. This finding was supported by a similar finding in the effect of DEX on FSHβ promoter activity and mRNA levels (**Figures 4.1.3-4.1.4 and Figures 4.2.2-4.2.3**). This was an expected result, given that previously published data showed DEX increased GnRH-induced FSHβ promoter-reporter

activity and mRNA levels (Thackray, McGillivray and Mellon, 2006; An *et al.*, 2009). The similarities in the increase of FSHβ expression from previous studies were likely due to the same concentration of 100 nM used for both DEX and GnRH, and the LβT2 cells were used as the model system (Thackray, McGillivray and Mellon, 2006; McGillivray *et al.*, 2007). Conversely, no effect by NET on basal and GnRH-induced FSHβ promoter-reporter activity or mRNA levels was detected. Similarly, no P4 effect on FSHβ expression was detected. These findings (**Figure 4.1.3-4.1.4 and Figure 4.2.2-4.2.3)** contrast with previously published data (Thackray, McGillivray and Mellon, 2006; McGillivray *et al.*, 2007). It is possible the effects by NET and P4 on FSHβ expression was undetected due to the lack of overexpression of PR-B, as the same concentrations of 100 nM of GnRH and P4 and cell line were used (Thackray, McGillivray and Mellon, 2006; An *et al.*, 2009).

Cga was investigated as it is one of the subunits of the protein structure for both LH and FSH (Fan and Hendrickson, 2005; Cahoreau, Klett and Combarnous, 2015). Similar to the β subunit of LH and FSH, there are no previous studies that have investigated the effects of MPA and NET on Cga mRNA. The decrease in Cga mRNA by DEX supports this decrease by MPA. This is similar to the decrease in GnRH-induced LHβ transcription by both MPA and DEX, but in contrast to the increased FSHβ expression by MPA and DEX (**Figures 4.2.1.1-4.2.1.2 and Figures 4.2.2-4.2.3**). The results on the effects on Cga mRNA levels by MPA do not provide enough information on whether MPA may regulate Cga levels as an additional mechanism to affect LH or FSH protein (**Figure 4.2.4**). Furthermore, the alpha subunit is made in excess as it forms part of multiple glycoprotein structures (Kourides *et al.*, 1980; Bernard *et al.*, 2010; Cahoreau, Klett and Combarnous, 2015). Additionally, literature indicates that changes occurring in LH and FSH levels are likely due to effects on changes in β subunit expression and protein synthesis (Kaiser *et al.*, 1997; Thackray, Mellon and Coss, 2010). Thus, it is likely that the decrease in Cga mRNA by MPA does not contribute to the mechanism used by MPA to regulate LH and FSH serum protein levels. Similar to the observations on LHβ and FSHβ expression by NET, the effects by NET were likely not detected due to lack of PR-B overexpression in the LβT2 cells.

5.4 GR is involved in mediating the effects by MPA and DEX on FSHβ expression in LβT2 cells.

MPA and NET can both act via the PR, while MPA, but not NET, can activate the GR (Ronacher *et al.*, 2009; Africander, Verhoog and Hapgood, 2011). The use of the GR agonist DEX and PR agonist P4 throughout this study indicated more typical GR-mediated, than PR-mediated regulation. These likely GR-mediated effects include the effect of DEX on down-regulation of the GnRH-induced LHβ promoter activity, Cga mRNA levels, and upregulation of basal and GnRH-induced FSHβ promoter activity and mRNA (**Figures 4.1.3-4.1.4 and Figures 4.2.24.2.3**). To the contrary, the only significant regulation detected with P4 was a decrease in LHβ promoter activity in the absence of GnRH (**Figure 4.1.2 A**). In all the in vitro experiments conducted in this thesis, MPA follows the same pattern, albeit to a lesser extent, of regulation as DEX (only in the case of FSHβ expression and Cga mRNA levels). The lower response to MPA compared to DEX may be because MPA likely acts as a partial agonist for the GR in the transactivation of FSHβ promoter activity via the GRE site (Ronacher *et al.*, 2009). This is likely as the FSHβ mouse promoter has a GRE site and DEX has been shown to increase mouse FSHβ promoter activity via direct binding of the GR to the GRE site in LβT2 cells (Thackray, McGillivray and Mellon, 2006). In the case of Cga mRNA levels, the lower response by MPA compared to DEX may be due to a lower potency of MPA rather than MPA being a partial agonist, given that there is repression of Cga. However, it would be expected that there would be saturation of the GR at 100 nM of MPA (Ronacher *et al.*, 2009). Therefore, the difference in the extent of decreased Cga mRNA levels by DEX and MPA, may be attributed to factors other than transrepression via the GR (Ronacher *et al.*, 2009).

While glucocorticoid activity for MPA has previously been shown in vivo in rats (Fekete and Szeberènyi, 1965), MPA has also been shown to have androgenic (AR) activity in the rat pituitary when rats did not express the PR-B protein (Pérez-Palacois *et al.*, 1983). Therefore, MPA could have activated the PR-B and/or AR. Unfortunately, an AR-specific agonist was not used as a control in the in vitro experiments. In this study AR-A, AR-B, PR-A and PR-B protein were not detected, although previous studies detected endogenous AR mRNA in LβT2 cells (O'Hara *et al.*, 2015) and PR mRNA was detected in this study, which was consistent with the literature (**Figures 4.4.1-4.4.1.2**) (Thackray, McGillivray and Mellon, 2006). In the current study, the only steroid receptor protein detected in LβT2 cells was the GR (**Figures 4.4.1.2**). Taken together, these results strongly suggest that MPA-induced regulation of the gonadotropins in this thesis was mediated by the GR. Given the fact that NET does not activate the GR (Ronacher *et al.*, 2009), the general lack of NET-induced regulation of the

gonadotropins is consistent with the undetectable expression of PR or AR protein in the LβT2 cells (**Figures 4.4.1.2 B and C**).

Lastly, the experiments involving the GR/PR antagonist RU486 also consolidate the involvement of the GR (**Figure 4.4.2**). The loss of the significant DEX and MPA-induced increase in GnRH-induced FSHβ promoter-reporter activity and mRNA levels in the presence of RU486 further supports the involvement of the GR. This is because RU486 is an antagonist and would inhibit the binding of DEX and MPA to the GR (Hapgood *et al.*, 2014) (**Figure 4.4.2**)**.** Previous research showed that FSHβ transcription is modulated by glucocorticoids via the direct binding of the GR to the endogenous FSHβ promoter in LβT2 cells (Thackray, McGillivray and Mellon, 2006). This aligns with the results seen in the RU486 experiments (**Figure 4.4.2 A**). This thesis is the first study to provide evidence of the involvement of GR in upregulation of FSHβ expression by MPA.

5.5 The effects of DMPA-IM and NET-EN on FSH, but not LH levels, likely occur in women via direct effects by the progestins on pituitary gonadotropes.

In the clinical samples, the use of DMPA-IM and NET-EN did not detectably change GnRH levels but decreased LH levels in women, suggesting that MPA and NET act directly on the pituitary to regulate LH levels. Unfortunately, in vitro results on the secreted LH protein did not support this hypothesis, since no significant changes in secreted LH with MPA or NET, in the presence of GnRH, were detected. An alternative explanation could be that DMPA-IM- and NET-EN-mediated regulation of LH levels in women does not occur at the pituitary level. While DMPA-IM and NET-EN did not detectably change serum GnRH levels, indicative of GnRH amplitude, it cannot be excluded that the progestins regulate LH levels by acting directly at a hypothalamic level or above to change GnRH pulsatility in women. However, it is also possible that MPA and NET do act directly on pituitary gonadotropes to regulate LH levels in vivo, but that the in vitro model does not retain all the components of the more physiologically relevant primary human pituitary gonadotrope cells, or that other signals, including paracrine effects in the pituitary, may be involved in vivo.

MPA and NET similarly activate PR and AR in vitro (Pérez-Palacois *et al.*, 1983; Stanczyk *et al.*, 2013; Africander, Storbeck and Hapgood, 2014). The decrease in LH levels in women by both DMPA-IM and NET-EN may be due to PR- or AR-mediated regulation of LH levels. While PR and AR protein were not detected in this study, these endogenous proteins have previously been detected in primary gonadotrope pituitary cells cultured from rats (Turgeon and Waring, 2000; Okada *et al.*, 2003). While there is evidence for the expression of endogenous AR/PR proteins in rat gonadotropes, there is no evidence for this in primary human gonadotropes. However, it is possible that AR/PR is expressed in human primary pituitary cells. Thus, it is possible that MPA and NET decrease LH directly in the gonadotropes via AR/PR mechanisms (if expressed) in women. In the other case, where AR/PR is expressed in other primary pituitary cell types but not in gonadotropes, MPA and NET may activate AR/PR mechanisms and exert paracrine effects on the gonadotropes to decrease LH in women. Additionally, the variation in the GR/AR/PR ratio in the gonadotropes and other pituitary cell types may affect the LH response to the progestins.

Interestingly, while MPA did not decrease LH secreted protein or LHβ mRNA levels, a decrease in GnRH-induced LHβ promoter-reporter activity was found in LβT2 cells. This could indicate that the similar GR-mediated transcriptional mechanism used in vitro (as discussed in section 5.3) is also occurring in vivo at the pituitary level in the LH protein decrease seen in the serum of DMPA-IM users (**Figures 3.2 and 4.1.2**). MPA may target the GnRH-induced transcription factor early growth response protein 1 (Egr-1), required for the transactivation of the LHβ promoter activity (Breen *et al.*, 2012). Egr-1 mRNA and protein are expressed in LβT2 cells (Windle, Weiner and Mellon, 1990). Additionally, the decrease seen in Cga mRNA in vitro likely does not contribute to the mechanism used by DMPA-IM to decrease LH levels in women. However, as mentioned above, there may be other post-transcriptional or secretory mechanisms that are not GR-mediated.

Interestingly, the increase in FSHβ gene expression by MPA in LβT2 cells is consistent with the increase in serum FSH protein levels observed in women on DMPA-IM (**Figures 3.2, 4.1.34.1.4 and 4.2.2-4.2.3**). However, although the secretion of FSH was not successfully investigated in vitro, it could still play a role in vivo. However, the regulation of FSH by steroidal hormones has been shown to occur more at the level of transcription rather than at the level of protein secretion in rodent primary pituitary cell cultures (McNeilly *et al.*, 2003; Bernard *et al.*, 2010;

Das and Kumar, 2018). Taken together, the findings from FSHβ regulation by MPA in vitro suggest that a transcriptional mechanism is most likely predominantly used by DMPA-IM to regulate FSH protein levels in vivo. Furthermore, the similarities between the clinical data and the in vitro data imply that DMPA-IM acts directly on pituitary gonadotropes in vivo in order to regulate FSH. The mechanism by which FSH serum levels are decreased in NET-EN users is unclear. The experiments in LβT2 cells suggest that the mechanism does not involve regulation of FSHβ at the level of transcription and mRNA expression by NET in pituitary gonadotropes. It could be speculated, given that NET-EN use decreased FSH levels in women and that AR and PR may be expressed in the human primary gonadotropes, that NET decreases FSHβ expression via direct actions on gonadotropes in vivo.

Although there are limitations in the in vitro model system, the fact that there are similarities maintained across different model systems (in vitro and in vivo) (O'Conner *et al.*, 1999; McNeilly *et al.*, 2003; Burger *et al.*, 2004; Nicol *et al.*, 2004; Thackray, McGillivray and Mellon, 2006; Thackray *et al.*, 2009; Breen *et al.*, 2012; Wang *et al.*, 2019; Capitaine, 2024) and different species (humans and mice) (Warren, Siris and Petrovich, 1977; Soules *et al.*, 1984; Lesoon and Mahesh, 1992; Thackray, McGillivray and Mellon, 2006; Breen *et al.*, 2012; Schliep *et al.*, 2015; Hutchens *et al.*, 2016; Capitaine, 2024) indicates the usefulness of LβT2 cells for investigating these mechanisms. Additionally, the results from this model system indicate that changes in response to DMPA-IM (but not particularly for NET-EN) are occurring directly at the pituitary gonadotrope level. This is an important gap that has been addressed. A representative model on the possible mechanism behind LH and FSH regulation by DMPA-IM and NET-EN (**Figures 5.1-5.2**).

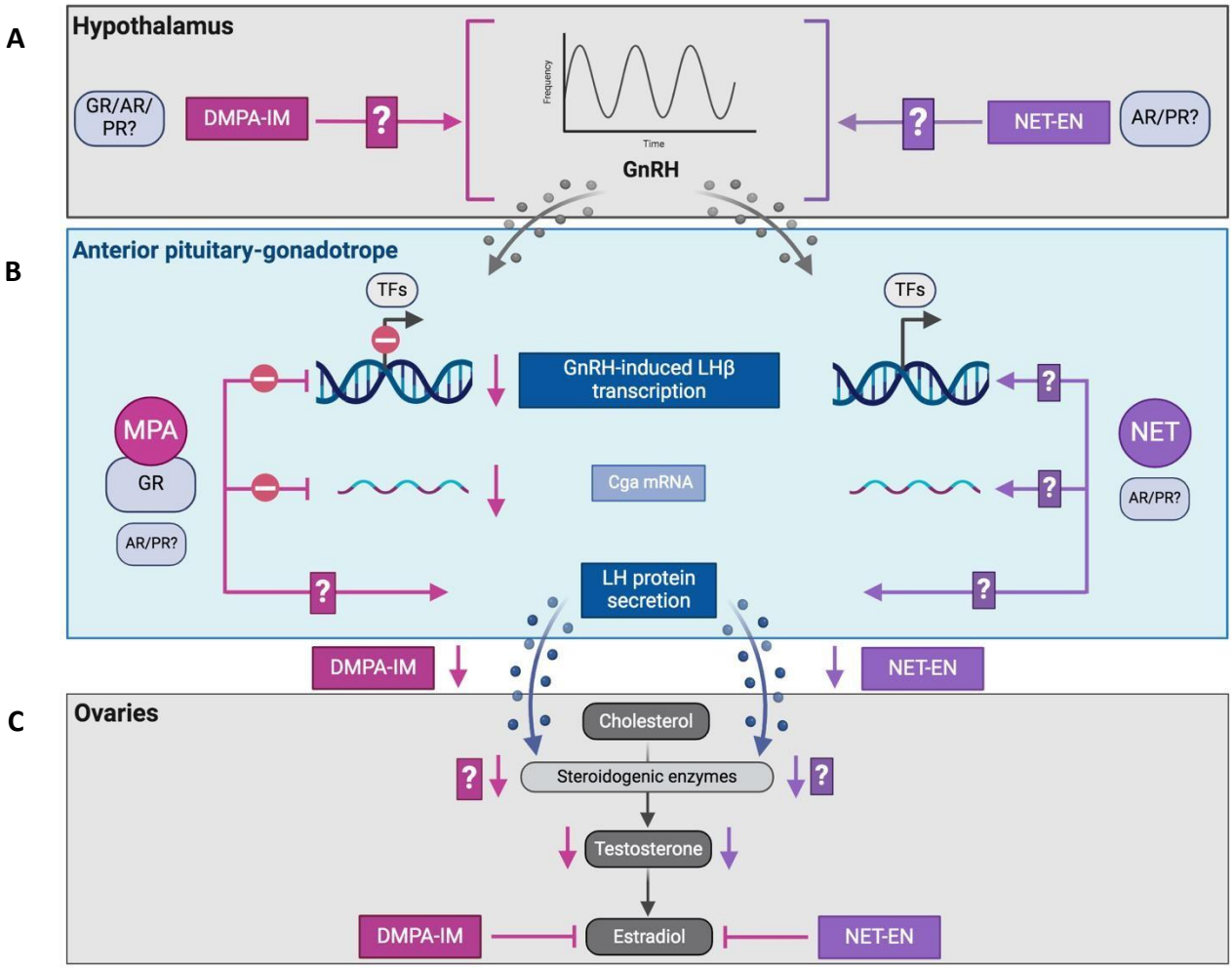


Figure 5.1. Model of the mechanism behind LH regulation by DMPA-IM and NET-EN and hypoestrogenism in women. *The model is based on the findings in LβT2 cells and clinical data presented in this thesis, as well as evidence from the literature. In* ***A****, although no effect of the contraceptives was detected in total serum GnRH levels, effects of DMPA-IM and NET-EN acting on the hypothalamus to change GnRH pulsatility may not have been detectable by this method. Since AR, PR and GR are all reportedly expressed in the hypothalamus in mammals (including women) in vivo, MPA could potentially act via all these SRs, while NET could act via both AR or PR, to cause possible effects on GnRH pulsatility. In* ***B****, both DMPA-IM and NET-EN decreased LH protein levels in women. The in vitro evidence from LβT2 cells showed that MPA acts directly on gonadotropes to decrease GnRH-induced LHβ transcription. Although MPA decreased Cga mRNA, it is unlikely that the decrease in the α subunit contributed to the decrease in LH protein secretion in women. Only the GR protein was detected in the LβT2 cells and thus it is likely that the decrease in LHβ transcription occurs at least in part via the GR on gonadotropes in women. However, it cannot be excluded that MPA could activate the AR and/or PR in the pituitary gonadotropes in women. MPA may also have an effect on LH protein*

secretion in women, but this could not be detected in the LβT2 cells. In women, NET-EN use may affect LHβ transcription, Cga mRNA and LH protein secretion via direct effects on the pituitary gonadotropes with NET acting via the PR or AR. In ***C,*** *the decrease in LH levels by DMPA-IM and NET-EN most likely decreases steroidogenesis in the ovary. This may explain the decrease in testosterone serum levels caused by DMPA-IM and NET-EN in women and thus the hypoestrogenic effect of these contraceptives. The data do not exclude possible additional direct effects of MPA and NET on the ovaries.*

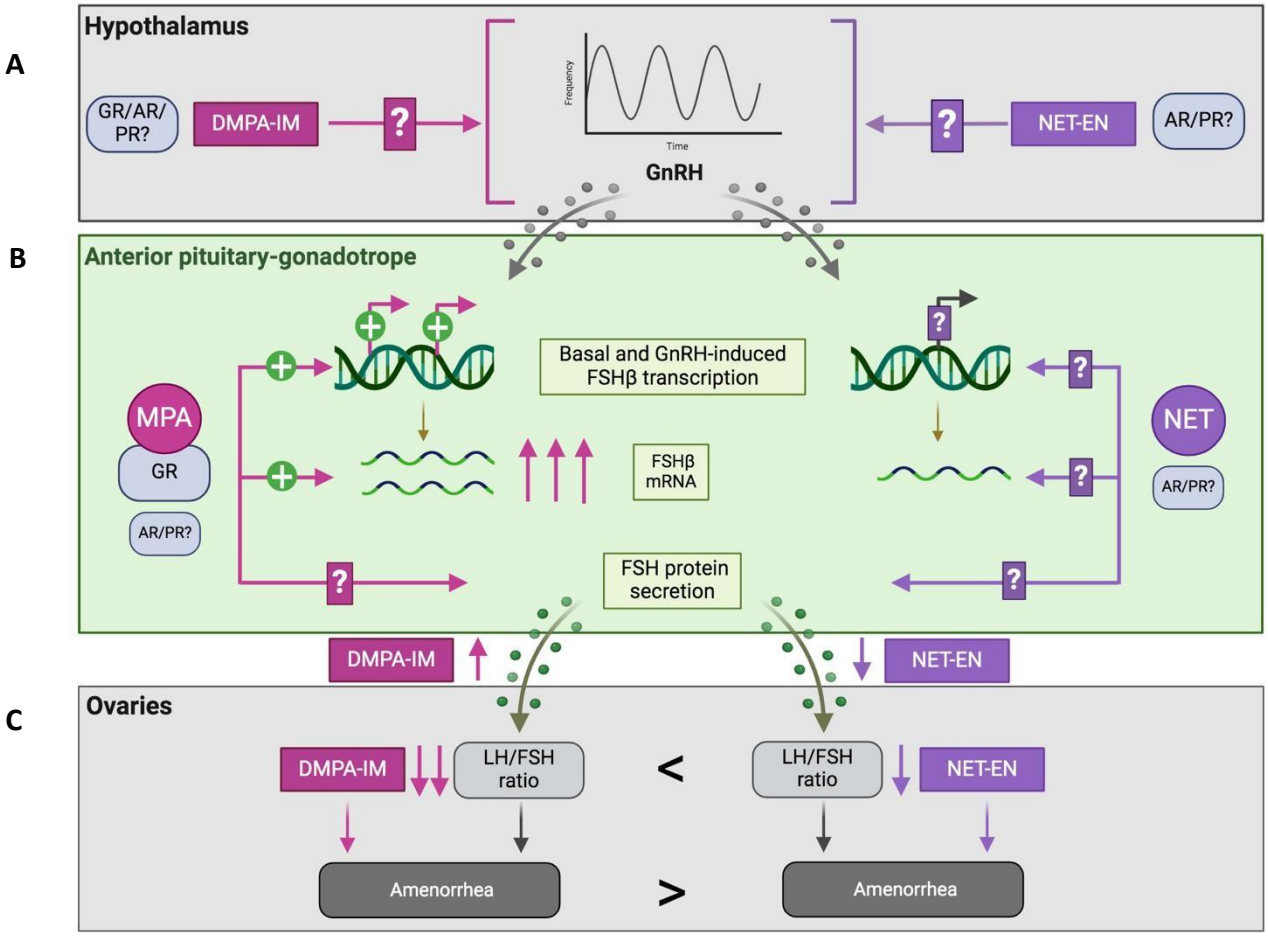


Figure 5.2. Model of the mechanism behind FSH regulation by DMPA-IM and NET-EN in women. *The model is based on findings in LβT2 cells and clinical data presented in this thesis, as well as evidence from the literature. In* ***A,*** *DMPA-IM and NET-EN may also affect GnRH pulsatility by the direct actions of the progestins at the hypothalamic level in order to cause the effects on FSH. In* ***B,*** *MPA has been shown to increase both FSHβ transcription and mRNA levels via the GR in LβT2 cells. However, it cannot be excluded that AR and PR may be expressed*

in the pituitary gonadotropes in women, and thus MPA may act via these steroid receptors to cause an increase in FSHβ expression. The increase in FSH levels in women by DMPA-IM is most likely due to a transcriptional mechanism used to increase FSHβ gene expression. However, MPA may also increase FSH protein levels via effects on protein secretion, but this was not tested in LβT2 cells. At the pituitary level, NET may affect FSHβ gene expression and FSH protein secretion via AR/PR, but this could not be detected in the
LβT2 cell model. However, FSH serum levels were decreased by NET-EN in women. In ***C,*** *the increase in FSH levels and decrease in LH levels (****Figure 5.1****) by DMPA-IM caused a decreased LH:FSH ratio in women to <1, compared to before injection. Additionally, the decreased LH (****Figure 5.1****) and FSH levels caused by NET-EN use in women also resulted in a decreased LH:FSH ratio to <1. However, DMPA-IM use caused a lower LH:FSH ratio compared to NET-EN use. Additionally, women on DMPA-IM exhibit more amenorrhea than those on NET-EN. An LH:FSH ratio of <1 has been associated with amenorrhea in women.*

5.6 Limitations

The WHICH clinical trial did not measure GnRH pulse frequency in women. GnRH serum levels were measured at initiation and 25W after the 6-month injection. Furthermore, this may not have detected changes in frequency of GnRH pulses and may also not have detected changes in amplitude of pulses, as this would depend on the turnover rate of GnRH. Thus, given this sampling method, the effects of the contraceptives on the frequency and amplitude of GnRH pulses may not have been detected. Furthermore, considering that the GnRH stimulation of the LβT2 cells was not given in pulses, the effects of the progestins in vitro may not accurately reflect the effects in vivo. The assay used for E2 serum measurements was not as sensitive compared to techniques such as mass spectrometry. Successful detection of secreted LH and FSH proteins has previously been reported for LβT2 cells (Thomas, Mellon, Turgeon, 1996; Graham, Nusser and Low, 1999). However, the assay used may not have the sensitivity to detect changes in LH/FSH secretion. Additionally, in the case of FSH secretion, the LβT2 cells may not secrete FSH protein without additional hormonal stimulation such as activin.

A further limitation is that experiments in the LβT2 cells only investigated the effects of stimulation with 100 nM of all ligands at a single time point. This concentration is higher than the peak serum concentrations of MPA and NET detected in the WHICH trial participants (Avenant *et al*., 2023). Additionally, the concentration of 100 nM used for GnRH in the LβT2 experiments may have not been optimal to detect changes in LHβ mRNA, since others reported detection of changes with 10 nM GnRH (Nicol *et al.*, 2002; Breen *et al.*, 2012). The use of time courses for stimulation and optimization of concentration curves may have revealed different and/or more robust results. Future in vitro studies should include more biological repetitions to increase the sample size, thus enhancing the likelihood of detecting significant changes that may have been missed due to low sample numbers. For example, the experiments on FSHβ expression have twice the sample number of the experiments done on the LHβ expression. Although more biological repetitions could have increased power, the limitations of time and the need for additional experiments prevented further biological repeats. Furthermore, the correction for different sites was not done in the statistical analysis of the clinical data. However, this was beyond the scope of the expertise of the candidate.

Lastly, a significant limitation of this study was that the cell model used may not have had all the necessary cellular machinery to mimic the effects of the progestins on gonadotropes in vivo (particularly in the case of NET-EN). As discussed above, it is unclear whether the LβT2 cells express the AR/PR protein, as the western blot may not be able to detect lower protein levels. Thus, this creates a limitation in detecting possible changes in LH and FSH expression by NET and P4. Additionally, the cell line and the gonadotropin promoter-reporter constructs used in this study were from rodents. There may be species-specific differences between rodent and human gonadotropes. However, it is worth mentioning that the promoter sequences of humans and mice show significant conservation, particularly in the conserved HRE sites (Thackray, McGillivray and Mellon, 2006; Fortin *et al.*, 2009). While the LβT2 cells used may not fully replicate all in vivo effects, there is a consistent increase in FSH by MPA in vitro and in vivo, which may be associated with relevant physiological effects in women.

5.7 Future work

There are numerous potential research directions to explore in this field in future. The effects of the progestins on GnRH pulsatility could be further investigated in mice, since this would be challenging in women. Additionally, to eliminate potentially confounding species-specific differences, the regulation of human Cga, LHβ and FSHβ promoter-reporter activity by MPA and NET could be investigated in LβT2 cells. The LβT2 cells either do not express AR and PR protein or express these steroid receptors at low levels. Given that the effects by NET and MPA on LH and FSH may likely be mediated by AR/PR, overexpression of these steroid receptors in the LβT2 cells could be done in the future. Thereafter, the same experiments could be repeated to measure the response of NET and MPA in the presence and absence of overexpressed PR and AR. In order to investigate the possible role of AR and/or PR protein in LβT2 cells, PR- or AR-specific antagonists could be used in combination with NET and MPA. More biological repetitions should be incorporated into future research to expand the sample size and improve the chance of detecting significant changes that the initial small sample size might have missed. Although, many studies do not culture the LβT2 cells in charcoal stripped serum DMEM, this would be advantageous to do in future work involving sex steroid regulation of the gonadotropins. This would eliminate any potential confounding effects of steroids present in FBS DMEM. Future studies should repeat estradiol measurements using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), which is considered the more analytically specific and sensitive method for quantifying circulating steroid hormones.

In order to further investigate whether the transcriptional mechanism by MPA includes recruitment of MPA-activated GR at the FSHβ promoter, a chromatin immunoprecipitation (ChIP) experiment could be conducted. Furthermore, an investigation of the collective impact of various hormones such as T and activin alone and in combination with the progestins, on the regulation of LH and FSH, could offer valuable insights. This may reveal synergic and crosstalk effects and may more closely mimic the hormonal milieu to which gonadotrope cells are exposed to in vivo. Lastly, whether the expression of steroidogenic enzymes in the ovary is influenced by LH and FSH in DMPA-IM and NET-EN users, could further expand on the mechanisms behind hypoestrogenism in injectable contraceptive users.

5.8 Conclusions

E2 has important reproductive functions as well as protective effects within the FGT, particularly in the context of viral infections such as HIV-1. While both DMPA-IM and NET-EN have been shown to result in hypoestrogenism, the mechanism(s) behind this is not known. This study showed for the first time that the use of DMPA-IM and NET-EN does not change GnRH levels, decreases LH levels, and differentially regulates, with DMPA-IM increasing and NET-EN decreasing, FSH levels in women. The hypoestrogenic effect of DMPA-IM and NET-EN is likely caused by the decrease in serum LH levels. A decrease in LH would decrease androgen levels and thus E2 production, since androgens are precursors for estrogens. While the effects of NET-EN in decreasing FSH levels, unlike DMPA-IM, may further prevent E2 synthesis, the physiological consequence of an increase in FSH with DMPA-IM may not affect hypoestrogenism. However, this increased FSH decreases the LH:FSH ratio, which may be linked to the higher incidence of amenorrhea in the DMPA-IM group compared to the NET-EN group. The in vitro data did support a mechanism whereby MPA may repress LH levels via direct action on pituitary gonadotropes. However, the in vitro data did not support such a mechanism for NET. This may be because the in vitro model and experimental conditions (such as undetectable levels of PR and AR protein) did not allow such a mechanism in human gonadotropes to be revealed, or that NET, and possibly also MPA, may decrease LH levels via other mechanisms, such as direct effects by MPA and NET at the hypothalamic level or above, to regulate GnRH pulsatility. The in vitro results are consistent with possible different mechanisms for MPA and NET in regulating LH levels in women. Nevertheless, the in vitro data did support a mechanism whereby the increase in FSH serum levels in women on DMPA-IM is likely to occur via direct effects of MPA on transcription of the FSHβ gene pituitary gonadotropes. Furthermore, this response is likely mediated by the GRs. Taken together, these findings provide valuable mechanistic insights into the regulation of gonadotropins by DMPA-IM and NET-EN. This is the only study that has observed the effects on LH and FSH of MPA and NET at the gonadotrope level in vitro, as well as compared these effects to those observed in women on DMPA-IM and NET-EN. The clinical findings in this thesis show that DMPA-IM and NET-EN have some similar but some different effects on the HPO axis, while the in vitro data

suggests differential effects and mechanisms for MPA and NET on gonadotropes. However, it is important to note that due to limitations of the cell line, the changes and mechanisms by NET could not be detected and thus future work will need to be done to consolidate these possible differential effects between NET and MPA. The physiological consequences of such differences are not immediately clear, but certainly do suggest that these two contraceptives should not be considered as being in the same contraception risk category for potential side-effects. Thus, it may be necessary for women to contemplate incorporating an E2 supplement with progestin-only contraceptives to counteract their hypoestrogenic effects.

Addendum A

Table A1: E2, LH and FSH concentrations in DMPA-IM and NET-EN users

DMPA-IM		
E2 (pmol/L)	**Age and Number of women**	**Reference**
79 (56-108) [3,6]	18-40; n=99	Singata-Madliki *et al.*, 2024
234 [1,2]	26-41; n=9	Jeppsson *et al.*, 1982
374 [1,2]	26-41; n=9	
342 [1,2]	26-41; n=7	
91.8-275.3 [2,4]	20; n=1	Jeppsson and Johansson, 1976
91.8-367 [2,4]	43; n=1	
80.8-201.9 [2,4]	24-42; n=11	Jeppsson *et al.*, 1977
321.2 (165.2) [1,3]	20-40; n=33	Siregar, Rita and Yusrawati, 2019
247.8 (175.8) [1,2,5]	26-40; n-value was not recorded	Toppozada, Parmar and Fotherby, 1978
132.2- 154.2 [1,2]	15-37; n=121	Mishell *et al.*, 1972
286.3 (91.8) [1,2]	Age not stated; n=24	Briggs and Briggs, 1972
146.8 [1,2]	Not stated in abstract	Mishell, 1996
230.5 (277.9) [1,2]	28; n=24	Schaffir, Isley and Woodward, 2010
69.4 (47.4) [1,2]	19-46; n=31	Clark *et al.*, 2001; Hickey, Marino and Tachedjian, 2016
97.6 (5.9) [1,2]	18-40; n=38	Miller *et al.*, 2000; Hickey, Marino and Tachedjian, 2016
LH (IU/L)	**Age and Number of women**	**Reference**
13.0[1,2]	26-41; n=9	Jeppsson *et al.*, 1982
13.0 [1,2]	26-41; n=9	
14.0 [1,2]	26-41; n=7	
5.0-15.0 [2,4]	20; n=1	Jeppsson and Johansson, 1976
10.0-20.0 [2,4]	43; n=1	
9.0 [2,4]	24-42; n=11	Jeppsson *et al.*, 1977
1.9 (0.7)-10.9 (3.3) [2,5]	26-40; n-value was not recorded	Toppozada, Parmar and Fotherby, 1978
1.8 (2.2-1.5) [2,5]	34-43; n=6	Perez-Lopez, L'Hermite and Robyn, 1975

5.5 (0.9) [2]	Age not stated; n=13	Goldzieher *et al.*, 1970
6.6 (0.6) [2]	Age not stated; n=36	
6.3 (0.4) [2]	Age not stated; n=46	
6.3 (0.5) [2]	Age not stated; n=61	
6.3 (0.5) [2]	Age not stated; n=48	
7.75 (0.7) [2]	Age not stated; n=41	
8.4 (0.6) [2]	Age not stated; n=38	
7.2 (0.31) [2]	Age not stated; n=31	
4.7 (0.9) [2]	Age not stated; n=6	
FSH (IU/L)	**Age and Number of women**	**Reference**
12.1 [1,2]	26-41; n=9	Jeppsson *et al.*, 1982
18.15 [1,2]	26-41; n=9	
17.6 [1,2]	26-41; n=7	
5.5-16.5 [2,4]	20; n=1	Jeppsson and Johansson, 1976
6.6-19.3[2,4]	43; n=1	
12.1 [1],[2]	24-42; n=11	Jeppsson *et al.*, 1977
7.61 (2.77) [3]	20-40; n=33	Siregar, Rita and Yusrawati, 2019
3.4(1.2)- 7.1(3.4) [2,5]	26-40; n-value was not recorded	Toppozada, Parmar and Fotherby, 1978
4.3 (4.7-4.0) [2,5]	34-43; n=6	Perez-Lopez, L'Hermite and Robyn, 1975
MPA (nmol/L)	**Age and Number of women**	**Reference**
6.8 (0.8)[1,2]	26-41; n=9	Jeppsson *et al.*, 1982 ; Bick *et al.*, 2021
5.17-9.06 [2,4]	20; n=1	Jeppsson and Johansson, 1976; Bick *et al.*, 2021
2.6-18.1[2,4]	28-32; n=8	Fotherby *et al.*, 1980; Bick *et al.*, 2021
3.88-18.1[1,2]	19-39; n=20	Fotherby and Koetsawang, 1982; Bick *et al.*, 2021
2.33-6.21[1,2]	18-40; n-448	Molatlhegi *et al.*, 2021; Bick *et al.*, 2021
24.3-99.6 [1,2]	Not stated; n=92	Koetsawang, 1977; Bick *et al.*, 2021
3.7[1,2]	18-34; n=38	Achilles *et al.*, 2018; Bick *et al.*, 2021
NET-EN		

[1] :Converted to SI units seen in table heading

E2 (pmol/L)	Age and Number of women	Reference
59.85 (69.5-87.23) [3,6]	18-40; n=94	Singata-Madliki *et al.,* 2024
132.9 (121.1) [1,2,5]	26-40; n-value was not recorded	Toppozada, Parmar and Fotherby, 1978
252 (190-234)[1,2,]	20-37; n=7	Landgren and Diczfalusy, 1980
617 (522-730) [1,2,4]	20-37; n=10	
183.5 -734.2 [1,2,4]	20-40; n=9	Goebelsmann *et al.*, 1979
411.5 (77.8)-662.6 (239.4) [1,2]	20-35; n=10	Saleh *et al.*, 1983
135.5 (119.27) [1,2]	32.6; n=73	Lawrie *et al.*, 1998
LH (IU/L)	**Age and Number of women**	**Reference**
1.6(0.6)-10.9 (3.7) [2,5]	26-40; n-value was not recorded	Toppozada, Parmar and Fotherby, 1978
10-30; 20-40 (peak values range) [2,4]	20-40; n=9	Goebelsmann *et al.*, 1979
2.8 (1.6) - 4.2 (2.3) [2]	20-35; n=10	Saleh *et al.*, 1983
8.09 (0.39) [2]	Age not stated; n=19	Garmendia, Kesserü and Llerena, 1973
12.4 (2.9) [2]	28-35; n=7	Anderson *et al.*, 1990
7.4 (1.6) [2]	28-34; n=7	
FSH (IU/L)	**Age and Number of women**	**Reference**
2.9 (1.3)-6.3 (2.0)[2,5]	26-40; n-value was not recorded	Toppozada, Parmar and Fotherby, 1978
<10 [2,4]	20-40; n=9	Goebelsmann *et al.*, 1979
1.14 (1.1)-2.25 (1.1) [2]	20-35; n=10	Saleh *et al.,* 1983
4.8 (0.8) [2]	28-35; n=7	Anderson *et al.*, 1990
3.9 (0.3) [2]	28-35; n=7	
NET (nmol/L)	**Age and Number of women**	**Reference**
13.4-30.16[1]	26-40; n=15	Toppozada, Parmar and Fotherby, 1978; Bick *et al.*, 2021
44.9 (18.1) [1,2]/ 40 (9.05)	20-40; n=9	Goebelsmann *et al.*, 1979; Bick *et al.*, 2021
28.48-65.67[1,2]	19-39; n=8	Fotherby *et al.*, 1980; Bick *et al.*, 2021
20.3 (10.9) [1,2]	19-38; n=6	Joshi *et al.*, 1989; Bick *et al.*, 2021
4.12[1,2]	18-34; n=41	Achilles *et al.*, 2018; Bick *et al.*, 2021
26.8-48.57[1,2]	Not stated; n=5	Weiner and Johansson, 1975; Bick *et al.*, 2021

[2] :mean (SD)/(SEM)

[3]:median (IQR)

[4]:Read off a graph

[5] : After 100ug GnRH injections given in 30 min intervals for 90 min and peak MPA concentrations

[6] : Data from sub-study of WHICH cohort

[7]: 150mg DMPA-IM ; 200mg NET-EN

Table A2: E2, LH and FSH concentrations in pre-menopausal and post-menopausal women

Pre-menopausal		
E2 (pmol/L)	**Age and Number of women**	**Reference**
275.3 (62.4)[1,2]	46 [2]; n= 1690	Randolph *et al.*, 2004
172.5 (14.7) (Early follicular phase), 249.6 (18.4) (Mid-follicular phase) and 598.4 (33) (Late follicular phase) [1,2]	20-34; n=23	Welt *et al.*, 1999
234.9 (36.7) (Early follicular phase), 385.5 (51.4) (Mid- follicular phase) and 881 (99.1) (Late follicular phase) [1,2]	35-46; n=21	Welt *et al.*, 1999
403.8 (33) (Early luteal phase), 521.3 (40.4) (Mid-luteal phase) and 374.4 (Late luteal phase) [1,2]	20-34; n=23	Welt *et al.*, 1999
488.2 (62.4) (Early luteal phase), 587.4 (73.4) (Mid-luteal phase) and 403.8 (73.4) (Late luteal phase) [1,2]	35-46; n=21	Welt *et al.*, 1999
362.6 (223.2) [1,2]	18-31; n=12	Hutchens *et al.*, 2016
211.2 (24.6) (Early follicular phase); 444.6 (65.3) (Late follicular phase); 575.6 (106.5) (Mid-luteal phase) [1,2]	Age not stated; n=5	Soules *et al.*, 1984
381.8 (356-407.6) [1,3]	45; n=555	Kaaks *et al.*, 2005
LH (IU/L)	**Age and Number of women**	**Reference**
5.46 (1.33) (early to mid follicular phase) [2]	20-30; n=6	Kluge *et al.*, 2012
3.7 (2.83 -5.9) [3] (Early follicular phase)	43.5 (41.5-46.5) [3]; n=21	Kawakita *et al.*, 2023
5.7 (2.7) [2] (Late follicular phase)	18-31; n=12	Hutchens *et al.*, 2016
6.14 (2.65) [2] (Early follicular phase)	20-29; n=129	Xu *et al.*, 2009
6.76 (2.03) [2] (Early follicular phase)	30-39; n=138	Xu *et al.*, 2009

10.4 (2.89) [2] (Early follicular phase)	40-49; n=154	Xu *et al.*, 2009
FSH (IU/L)	**Age and Number of women**	**Reference**
19.5 (0.4) [2]	46.4 (2.7) [2]; n=1690	Randolph *et al.*, 2004
8 (0.4) [2]	23.5 (0.4) [2]; n=13	Klein *et al.*, 1996
5.97 (0.57) (early-mid follicular phase) [2]	25.5 (2.9) [2]; n=6	Kluge *et al.*, 2012
11.2 (0.7) (early follicular phase), 11.3 (0.6) (mid follicular phase) and 9.2(0.6) (late follicular phase) [2]	20-34; n=23	Welt *et al.*, 1999
13 (0.5) (early follicular phase), 11.4 (0.8) (mid follicular phase) 8.8 (0.5) (late follicular phase) [2]	35-46; n=21	Welt *et al.*, 1999
9.7 (0.6) (early luteal phase) 6.4 (0.5) (mid luteal) 6.2 (0.4) (late luteal phase) [2]	20-34; n=23	Welt *et al.*, 1999
12.5 (1) (early luteal phase) 6.8 (0.6) (mid luteal phase) 7 (0.4) (late luteal phase) [2]	35-46; n=21	Welt *et al.*, 1999
17.8 (13.1) [2]	45.6 (2.7)[2]; n=543	Sowers *et al.,* 2007
5.6 (4.8-8) [3]	43.5 (41.5-46.5)[3]; n=21	Kawakita *et al.*, 2023
3.5 (1.0) [2]	18-31; n=12	Hutchens *et al.*, 2016
2.81 (1.6) [2]	20-29; n=129	Xu *et al.*, 2009
2.98 (1.41) [2]	30-39; n=138	Xu *et al.*, 2009
7.33 (2.72) [2]	40-49; n=154	Xu *et al.*, 2009
9.9 (3.9) [2]	45-51; n=17	Kalleinen *et al.*, 2008
3.5 (1.0) [2] (Late follicular phase)	18-31; n=12	Hutchens *et al.*, 2016
5.87 (0.5) [2]	25.5; n=6	Kluge *et al.*, 2012
Post-menopausal		
E2 (pmol/L)	**Age and Number of women**	**Reference**
74.1 (125.1) [1,2]	40-65; n=1015	Ausmanas *et al.*, 2007
32.89 (54.23) (6-10 months)*, 12.89 (28.38) (12-21.6 months) * 7.34 (3.79) (22.8-36 months)* [1,2]	52.45 (2.49)[2]; n=146	Kling *et al.*, 2019

45.8 (18.4-106.5) [1,3]	51 (49.5-53) [3]; n=30	Kawakita *et al.*, 2023
45.8 (18.4-126.6) [1,3]	53 (51.75-54)[3]; n=42	Kawakita *et al.*, 2023
158 (25.7) [1,2]	40-56; n=127	Grub *et al.*, 2021
LH (IU/L)	**Age and Number of women**	**Reference**
36.8 (15.6)[2]	40-65; n=1015	Ausmanas *et al.*, 2007
37.4(16.82) (6-10 months)* 39.2 (15.54) (12-21.6 months)* 40.2 (17.05) (22.8-36 months)* [2]	52.45 (2.49)[2]; n=146	Kling *et al.*, 2019
8.8 (7.7-26.3) [3]	47 (46-49)[3] ; n=23	Kawakita *et al.*, 2023
30.05 (17.48-35.78)[3]	49 (46.5-50.5)[3] ; n=35	Kawakita *et al.*, 2023
33.3 (20.8-43.9) [3]	51 (49.5-53)[3] ; n=30	Kawakita *et al.*, 2023
30.55 (23.5-38.48) [3]	53 (51.75-54) [3]; n=42	Kawakita *et al.*, 2023
29.1(8.4) [2]	50-65; n=11	Baccarelli *et al.*, 2001
25.1(13.9) [2]	65-90; n=12	Baccarelli *et al.*, 2001
19.6 (12.7)[2]	90-95; n=10	Baccarelli *et al.*, 2001
9.9 (9.8) [2]	95-104; n=10	Baccarelli *et al.*, 2001
32.8 (1.88) [2]	50-59; n=133	Xu *et al.*, 2009
21.9 (1.86) [2]	60-69; n=124	Xu *et al.*, 2009
13.9 (1.70) [2]	>70; n=21	Xu *et al.*, 2009
FSH (IU/L)	**Age and Number of women**	**Reference**
11.4 (0.5) [2]	42.3 (0.6) [2] ; n=10	Klein *et al.*, 1996
81.2 (31.4) [2]	40-65; n=1013	Ausmanas *et al.*, 2007
76.27 (32.39) (6-10 months)*, 83.52 (31.60) (12-21.6 months)* 95.33 (35.02) (22.8-36 months)* [2]	52.45 (2.49) [2]; n=146	Kling *et al.*, 2019
23.9 (13.5-42.9) [3]	47 (46-49) [3] ; n=23	Kawakita *et al.*, 2023
56.95(30.55-87.35) [3]	49 (46.5-50.5) [3]; n=35	Kawakita *et al.*, 2023
93.4 (70.7-110) [3]	51 (49.5-53) [3]; n=30	Kawakita *et al.*, 2023
95.85 (71.43-123.7) [3]	53 (51.75-54) [3]; n=42	Kawakita *et al.*, 2023
71 (24.8)[2]	50-65; n=11	Baccarelli *et al.*, 2001

55.9 (30.2) [2]	65-90; n=12	Baccarelli *et al.*, 2001
45.2 (20.2) [2]	90-95; n=10	Baccarelli *et al.*, 2001
25.9 (23.5) [2]	95-104 ; n=10	Baccarelli *et al.*, 2001
33.4 (17.5) [2]	50-59; n=133	Xu *et al.*, 2009
26.9 (8.4) [2]	60-69; n=124	Xu *et al.*, 2009
17.8 (3.9) [2]	>70; n=21	Xu *et al.*, 2009

[1] :Converted to SI units seen in table heading

[2] :mean (SD)/(SEM)

[3]:median (IQR)

[*]:Time since onset of menopause

Table A3: Concentrations of the regulators of LH and FSH in pre and post-menopausal women

Pre-menopausal		
GnRH (pg/mL)	**Age and Number of women**	**Reference**
16.5 (0.5) (Follicular phase)[2]	Age not stated; n=5	Sarda, Barnes and Nair, 1981
19.8 (1.1) (Luteal Phase) [2]	Age not stated; n=5	Sarda, Barnes and Nair, 1981
Testosterone (nmol/L)	**Age and Number of women**	**Reference**
0.54 (0.23) [2]	18-31; n=12	Hutchens *et al.*, 2016
0.72(0.05) (early follicular phase); 1.05 (0.06) (midcycle peak); 0.81(0.06) (mid-luteal phase)[2]	<50; n=14	Miller *et al*., 2001
1.66 (1.6-1.72) [3]	45.6; n=555	Kaaks *et al.*, 2005
P4 (nmol/L)	**Age and Number of women**	**Reference**
1.3 (0.29) [2]	18-31; n=12	Hutchens *et al.*, 2016
1.0 (0.1) (early follicular phase); 1.0 (0.1) (late follicular phase); 23.8 (mid-luteal phase) [2]	Age not stated; n=5	Soules *et al.*, 1984
11.96 (10.59-13.34) (luteal phase)[3]	45.6; n=555	Kaaks *et al.*, 2005
Post-menopausal		
GnRH (pg/mL)	**Age and Number of women**	**Reference**
23.5 (0.2) -1743.2 (97.7)[2]	Age not stated; n=4	Sarda, Barnes and Nair, 1981

Testosterone (nmol/L)	Age and Number of women	Reference
0.9 (0.08)[2]	>50 ; n=14	Miller *et al*., 2001
P4 (nmol/L)	**Age and Number of women**	**Reference**
0.2 (0.28) [2]	40-56; n=127	Grub *et al.*, 2021

[1] :Converted to SI units seen in table heading

[2] :mean (SD)/(SEM)

[3]:median (IQR)

Table A4: Concentrations of the regulators of LH and FSH in women using DMPA-IM and NET-EN

DMPA-IM		
Testosterone (nmol/L)	**Age and Number of women**	**Reference**
0.423 (0.281-0.61)[3]	18-40; n=215	Avenant *et al*., 2024
1.8	26-41; n=9	Jeppsson *et al.*, 1982
1.8	26-41; n=9	Jeppsson *et al.*, 1982
2.0	26-41; n=7	Jeppsson *et al.*, 1982
0.1 (0.07)	Age not stated; n=24	Briggs and Briggs, 1972
1.2 (0.5)	28; n=24	Schaffir, Isley and Woodward, 2010
P4 (nmol/L)	**Age and Number of women**	**Reference**
<2.0 [2]	26-41; n=9	Jeppsson *et al.*, 1982
<2.0[2]	26-41; n=9	Jeppsson *et al.*, 1982
<2.0 [2]	26-41; n=7	Jeppsson *et al.*, 1982
<1.0[2,4]	20; n=1	Jeppsson and Johansson, 1976
<1.0[2,4]	43; n=1	Jeppsson and Johansson, 1976
<0.75[1,2]	20-25; n=444	Molatlhegi *et al.*, 2021
2.9 (4)[1,2]	20-4-; n=33	Siregar, Rita and Yusrawati, 2019
1.9 (1) [1,2]	Age not stated; n=24	Briggs and Briggs, 1972
<1.27[1,2]	Not stated in abstract	Mishell, 1996
1.3 (0.9 -1.4) [1,3]	18 years and older; n=8	Boodhram *et al.*, 2019

1.27 (0.6) [1,2]	19-46; n=31	Clark *et al.*, 2001; Hickey, Marino and Tachedjian, 2016
NET-EN		
Testosterone (nmol/L)	**Age and Number of women**	**Reference**
0.253 (0.086-0.385)[3]	18-40; n=219	Avenant *et al*., 2024
1.08 (2.38) [2]	32.6; n=73	Lawrie *et al.*, 1998
P4 (nmol/L)	**Age and Number of women**	**Reference**
<1.4 (peak value during 1 cycle); <1.4 (luteal phase)[2,4]	20-37; n=7	Landgren and Diczfalusy, 1980
<1.4 (peak value during 1 cycle); <1.4 (luteal phase) [2,4]	20-37; n=10	Landgren and Diczfalusy, 1980
13.7 (peak value during 1 cycle); 4.8 (luteal phase) [2,4]	20-37; n=9	Landgren and Diczfalusy, 1980
45.6 (peak value during 1 cycle); 22.6 (luteal phase) [2,4]	20-37; n=17	Landgren and Diczfalusy, 1980
<6.36; 12.7-22.3 (peak value)[1,4]	Age not stated; n=9	Goebelsmann *et al.*, 1979
4.1 (0.1)-5.4(0.1) [2]	20-35; n=10	Saleh *et al.*, 1983
0.7 (3.61) [2]	32.6; n=73	Lawrie *et al.*, 1998

[1] :Converted to SI units seen in table heading

[2] :mean (SD)/(SEM)

[3]:median (IQR)

[4]: Read off graph

[5]: 150mg DMPA-IM ; 200mg NET-EN

Addendum B

All experiments on serum samples and subsequent data collection were done by senior members in the Hapgood lab or external laboratories. The analyses on the data collected from the WHICH clinical trial were done by the candidate.

Chemiluminescent microparticle immunoassay (CMIA)

E2 from the serum samples of the WHICH participants was measured using CMIA (ARCHITECT Estradiol B7K720, analytical sensitivity ≤ 10 pg/mL). This assay was done on serum collected at the baseline and at the 25W time points. The assay was conducted by Neuberg Global Laboratories (Durban, KwaZulu Natal, South Africa) (Singata-Madliki *et al.*, 2024).

Electrochemiluminescence immunoassay (ECLIA)

LH and FSH were measured from serum samples taken from participants under the WHICH trial and measured using the sandwich-ELICA method. The Roche Elecsys and cobas e 601 & 602 immunoassay analyzer system was used to perform this method. The NHLS services (Groote Schuur hospital, Cape Town, South Africa) were used to conduct this assay (Singata-Madliki *et al.*, 2024).

ELISA

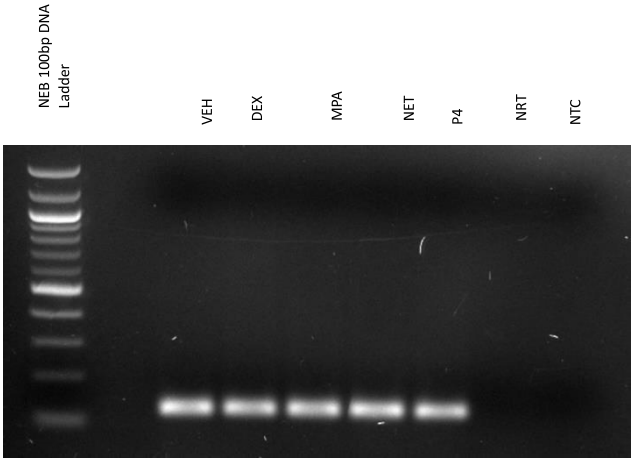
A GnRH ELISA kit (Elabscience Biotechnology Co., USA, catalogue no. E-EL-0071) was used to measure GnRH in serum samples according to the manufacturer's instructions. The GnRH ELISA was conducted by senior Hapgood lab members (Dr Chanel Avenant, Dr. Alexis Bick, Dr Johnson Mosoko Moliki and Miss Gcina Dlamini).

Addendum C

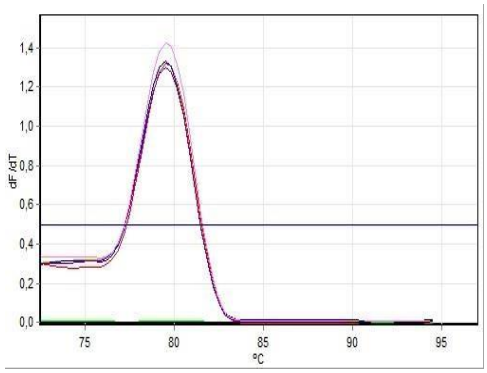
1 Important findings from troubleshooting

1.1 TBP is a more reliable house keeping gene than GAPDH in LβT2 cells, as GAPDH has multiple isoforms that are detected in a no reverse transcriptase control (NRT)

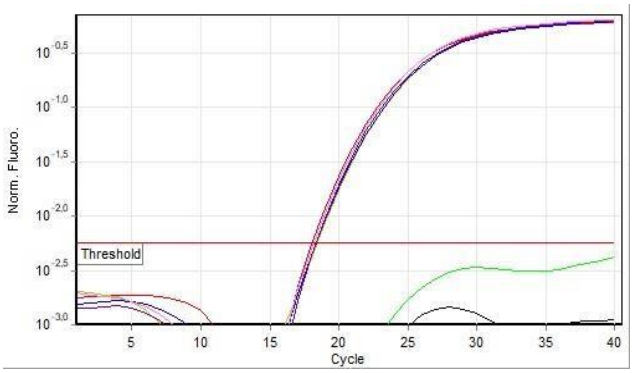
A



B



C

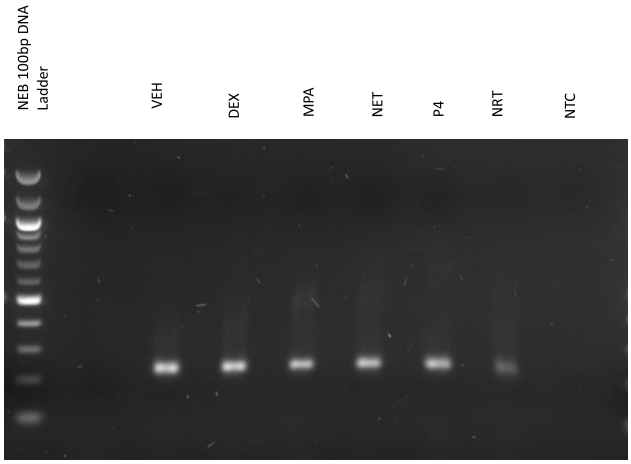


D

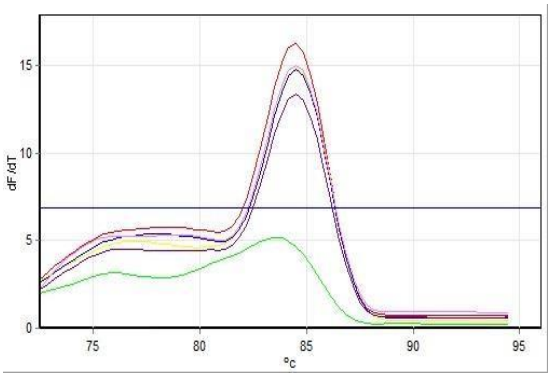
No.	Colour	Name	Ct
1		VEH	18,01
2		DEX	18,42
3		MPA	18,32
4		NET	18,31
5		P4	18,14
6		NRT	
7		NTC	

Figure A1.1.1 TBP RT-qPCR. *L*βT2 cells were grown for 24 hours prior to treatment with steroid ligands. The cells were treated with 100 nM of each steroid ligand and ethanol (vehicle, VEH) for 8 hours. Subsequently, RNA was isolated and cDNA was synthesized. RTqPCR was done to measure TBP mRNA and the RT-qPCR products of 132 bp were visualized on a 2% agarose gel using gel electrophoresis (A) and each product was confirmed using melt curve analysis (B). NRT and NTC defines the no reverse transcriptase and no template control. C_T values were calculated using Rotor Gene 6000 Corbett™ software analysis (C) with C_T values represented in (D).

A



B



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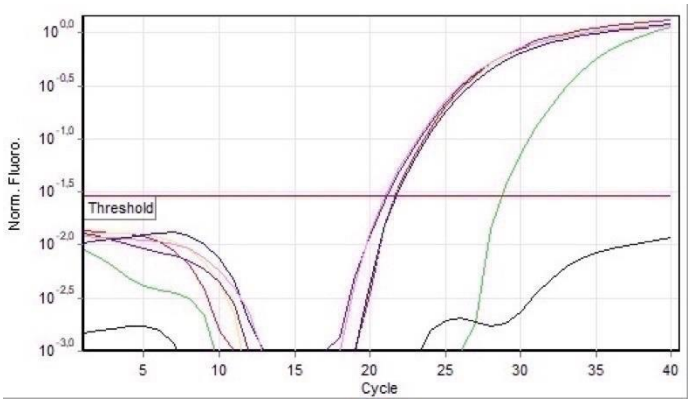


D

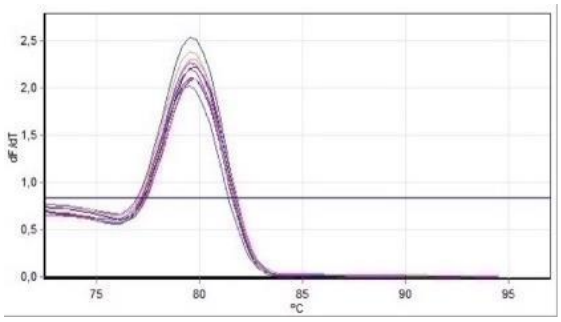
No.	Color	Name	Ct
1		VEH	21,67
2		DEX	21,74
3		MPA	21,75
4		NET	21,17
5		P4	21,04
6		NRT	28,75
7		NTC	

Figure A1.1.2. GAPDH RT-qPCR. LβT2 cells were grown for 24 hours prior to treatment with steroid ligands. The cells were treated with 100 nM of each steroid ligand and ethanol (vehicle)

for 8 hours. Subsequently, RNA was isolated and cDNA was synthesized. RT-qPCR was done to measure GAPDH mRNA and the RT-qPCR products of 132 bp were visualized on a 2% agarose gel using gel electrophoresis (A) and each product was confirmed using melt curve analysis (B). NRT and NTC defines the no reverse transcriptase and no template control. C_T values were calculated using Rotor Gene 6000 Corbett^{TM} software analysis (C) with C_T values represented in (D).

1.2 Older passage numbers in LβT2 cell stocks do not reliably express FSHβ mRNA

A



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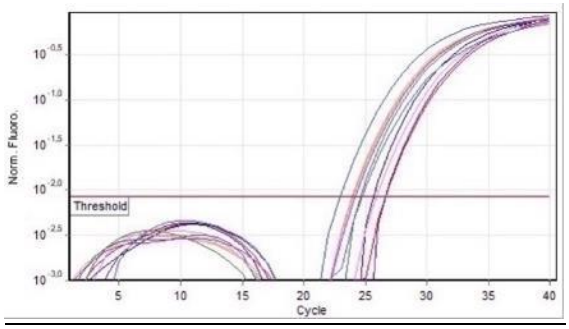
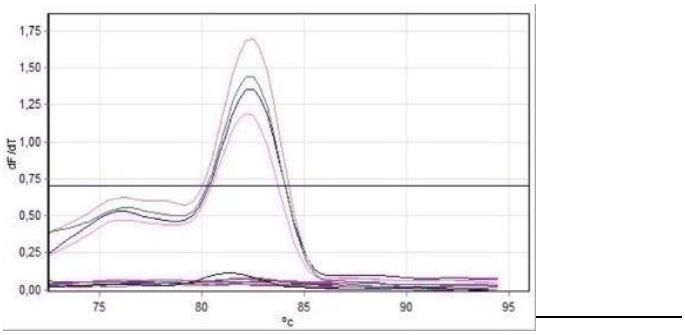


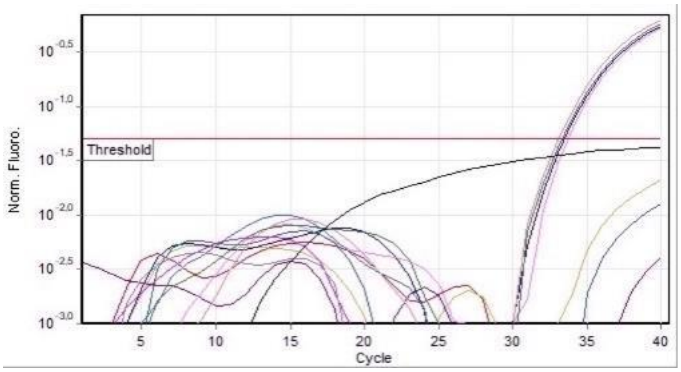
C

No.	Name	Ct
1	VEH	26,59
2	DEX	23,84
3	MPA	25,58
4	NET	26,59
5	P4	25,86
6	GNRH	24,68
7	DEX/GNRH	22,89
8	MPA/GNRH	23,93
9	NET/GNRH	24,54
10	P4/GNRH	24,12
11	NTC	

D



E



F

No.	Name	Ct
1	VEH	
2	DEX	
3	MPA	33,43
4	NET	
5	P4	33,68
6	GNRH	
7	DEX/GNRH	33,05
8	MPA/GNRH	33,28
9	NET/GNRH	
10	P4/GNRH	
11	NTC	

Figure A1.2.1. FSHβ RT-qPCR. *LβT2 cells were grown for 24 hours prior to treatment with steroid ligands. The cells were treated with 100 nM of each steroid ligand and ethanol (vehicle) for 8 hours. Subsequently, RNA was isolated and cDNA was synthesized. RT-qPCR was done to measure FSHβ mRNA. FSHβ RT-qPCR was done in cDNA from p17 LβT2 cells (A-C) and p31 LβT2 cells (D-F). Each product was confirmed using melt curve analysis (A and D). NRT and NTC defines the no reverse transcriptase and no template control. C_T values were calculated using Rotor Gene 6000 Corbett™ software analysis (B and E) with C_T values represented in (C and F).*

2 Supplementary data

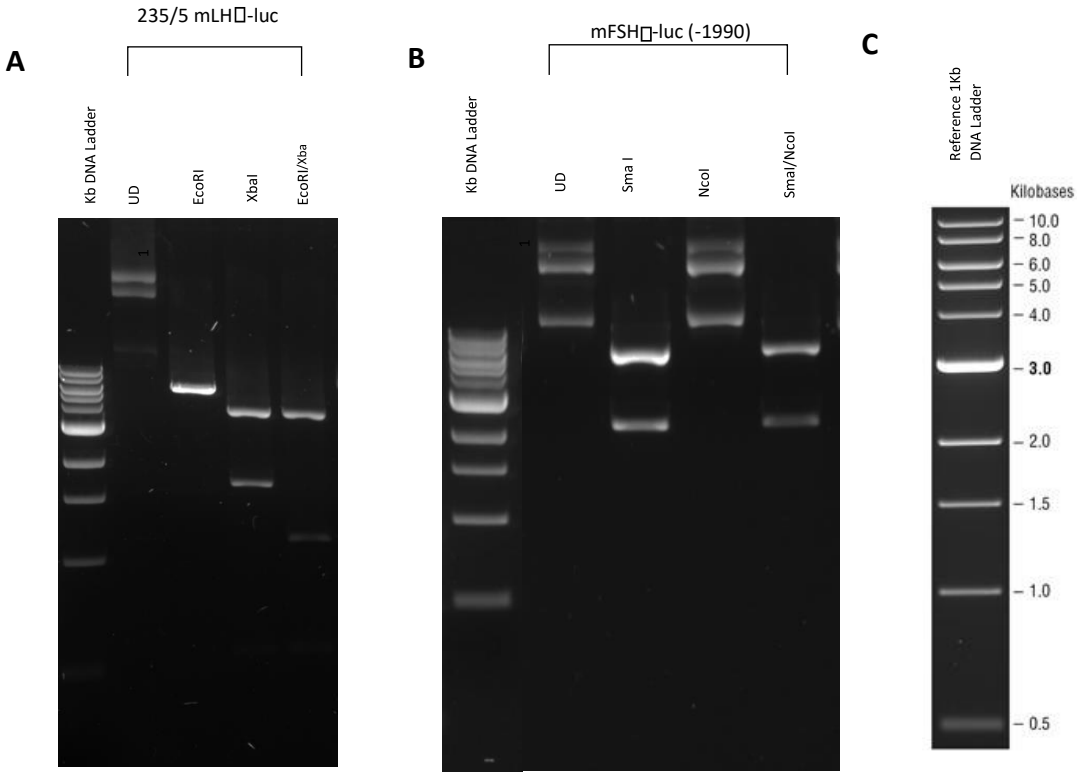


Figure S1: Representation of the restriction enzyme digests on DNA promoter-reporter constructs. *E.coli cells were transformed with either -235/5 mLH*β*-luc (**A**), mFSH*β*-luc (-1990) (**B**) plasmids and plasmid DNA was purified using the Nucleo-bond Xtra Maxi DNA purification kit. Thereafter the inserts in the purified plasmid DNA were confirmed by using the appropriate restriction enzymes (RE) in **A-B.** A RE digest was conducted and the products from the digests were visualized using 1.25% agrose gel electrophoresis. In **C,** a representation of a reference1Kb NEB DNA ladder used during gel electrophoresis is shown.*

Table S1: Expected sizes of restriction enzyme digest of plasmid DNA

Plasmid DNA	Expected restriction enzyme digest of plasmid DNA sizes (bp)			
	Uncut	EcoRI	XbaI	EcoRI + XbaI
-253/5 mLHβ	Supercoiled<6587 Nicked>6587	6587	1655 & 4932	4932,1115,540
Plasmid DNA	**Uncut**	**SmaI**	**NcoI**	**SmaI/NcoI**
mFSHβ-luc (1990)	Supercoiled<6809 Nicked>6809	4818, 1991	6809	4760,1991,58

Table S2: Observed sizes of restriction enzyme digest of plasmid DNA

Plasmid DNA	Observed restriction enzyme digest of plasmid DNA sizes (Kb)			
	Uncut	EcoRI	XbaI	EcoRI + XbaI
-253/5 mLHβ	Supercoiled<6587 Nicked>6587	6000<8000	4000<5000 1500<2000	4000<5000 1000<1500 500<1000
Plasmid DNA	**Uncut**	**SmaI**	**NcoI**	**SmaI/NcoI**
mFSHβ-luc (1990)	Supercoiled<6809 Nicked>6809	4000<5000 1500<2000	Did not cut Supercoiled<6809 Nicked>6809	4000<5000 1500<2000

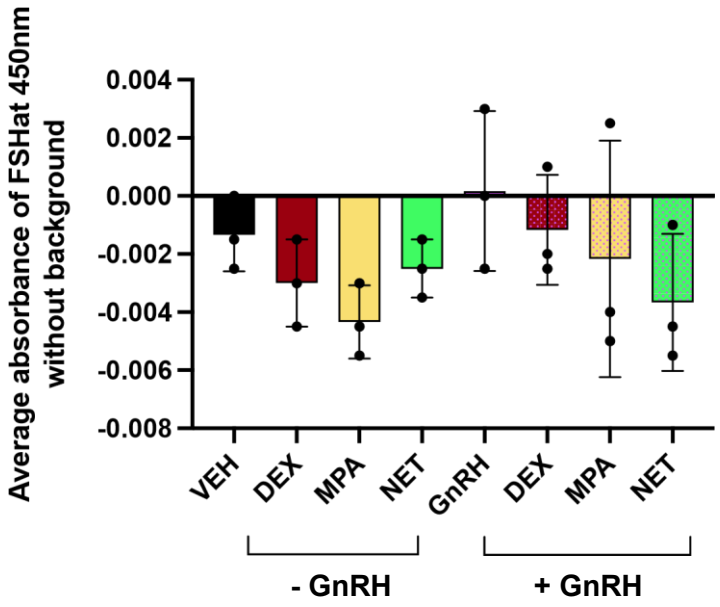


Figure S2: Mouse FSH ELISA kit cannot detect FSH secreted protein. *LβT2 cells were grown overnight and stimulated with 100 nM of either steroid ligands or 0,1% ethanol and sterile water (v/v) (vehicle) for 8 hours. 100 nM GnRH was added alone and in combination with the other steroid ligands during the last 6 hours. Following the stimulation, supernatant media was collected. A mouse FSH ELISA kit (section* ***2.8****) was used to measure FSH secretion as per the manufacturer's instructions. The background absorbance of the dilution solvent used for the supernatant was subtracted from total absorbance measured.*

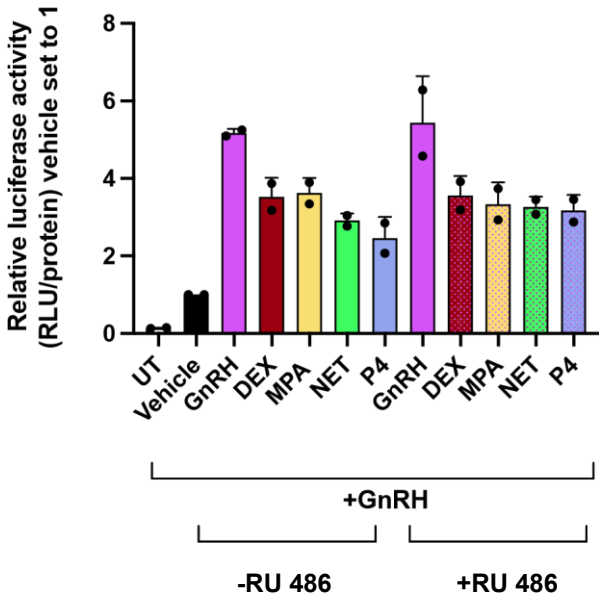


Figure S3: GR may not be involved in the possible decrease of GnRH-induced LHβ promoter activity by MPA. *LβT2 cells were seeded, transfected and stimulated with 100nM of RU486 and the selected ligands as per Figure S3. 100 nM GnRH was added to the cells alone or to cells already stimulated with another steroid ligand, for the last 6 hours, prior to cell harvesting. Luciferase activity was measured and normalized against protein concentration of each well. The data is plotted as the normalized luciferase activity fold induction relative to GnRH in the absence of RU486, that is set to 1.*

Addendum D

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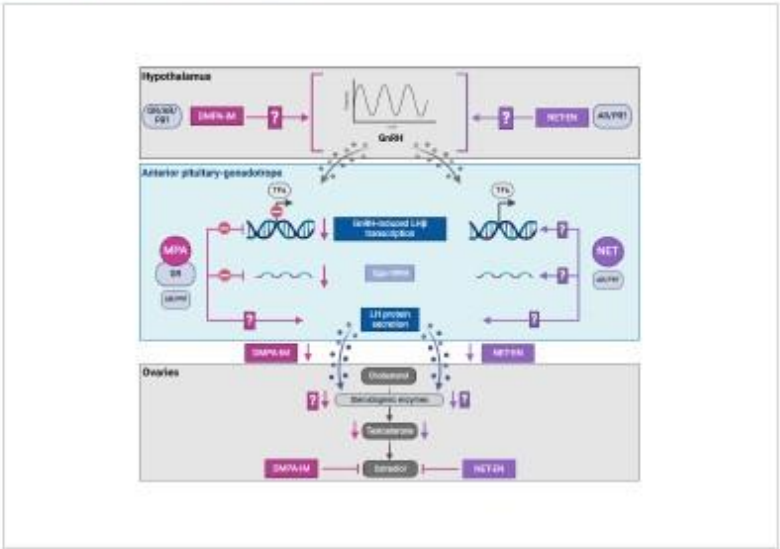

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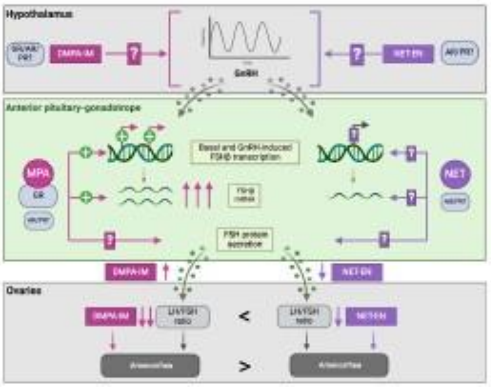

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