In vitro endocrine modulation within the Hypothalamus-Pituitary-Ovary (HPO) axis by dioxin like compounds and phytoestrogens

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In vitro endocriene modulatie in de Hypothalamus-Hypofyse-Ovarium (HHO) as

door dioxineachtige verbindingen en fyto-oestrogenen
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

CHAPTER 1

General Introduction

1. Female reproduction

A key function of reproduction is the perpetuation of the species. The female reproductive cycle is one of the most complex sets of endocrine processes that takes place in the mammalian body. The female reproductive system consists of the ovaries, oviducts, uterus and vagina, and it is under control of a precise network of regulatory signals that are neuro- and hormonally regulated by the hypothalamus, pituitary and ovaries. These interactions are arranged within the so-called hypothalamus – pituitary – ovary (HPO) axis [1; 2; Fig.1]. All these organs function in conjunction to fulfill the main roles of the female reproductive system; producing the female sex hormones and generate viable gametes to promote fertilization and support pregnancy. A hallmark of the female reproductive axis is its cyclic nature that results in ovulation. This is preceded by complex hormonal events such as a pre-ovulatory rise in estradiol (E2) secretion by dominant follicles of the ovary followed by the pre-ovulatory surge of gonadotropins [3]. In adult females, the ovaries are mainly responsible for the production of the sex steroid hormones, but they are also the main source of germ cells. This female reproductive process is also under control of negative and/or positive hormonal feedback mechanisms, depending on the physiological state of the female [4; 5].

Rodents have widely been used as animal models to study the physiology of the reproductive axis [6]. Their short reproductive cycle and life span facilitate studies on the reproductive cyclicity. From a legislative point of view there is also a requisite for an animal model to test reproductive and developmental toxicity, such as the one-generation reproduction toxicity study (OECD TG 415), extended one-Generation Reproductive Toxicity Study (OCED TG 443), two-generation reproduction toxicity study (OECD TG 416) and the reproductive/developmental toxicity screening assay (OECD TG 421). Some reproductive and developmental differences between rodents and humans exist, such as the number of ovulations and length of the cycle, nonetheless the reproductive function in all mammals rely on a tight cascade of events that originates from gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus. The anatomical organization of the GnRH neuronal system in humans differs somewhat from rodents. The GnRH perikarya in humans occupy two major regions: half of the neurons are located in an anterior region that involves the preoptic-septal region and the other half in a posterior region involving the infundibulum (arcuate nucleus in rodents). In rodents, over 90% of gonadotropin-releasing hormone-immunoreactive (GnRH-IR) perikarya are located rostrally in the diagonal band of Broca, medial septum, and medial preoptic area. In spite of these differences, experimental studies suggest similar innervation pattern of GnRH in humans and rodents [7]. Under physiological conditions, metabolic-, stress-, neuronal-, sex steroid- and circadian signals regulate GnRH neurons directly or act on upstream neuronal circuitries to control the pattern of pulsatile GnRH secretion into the hypophysial portal circulation. This pulsatile pattern of GnRH release is essential for normal ovarian function. In the female, this strong release of GnRH generates the *luteinizing hormone* (LH) surge, which is a prerequisite for the ovulation process. In addition, the driving force behind this GnRH/LH synthesis and

secretion also relies on a complex balance between stimulatory and inhibitory inputs of multiple neurotransmitters, like Gamma-aminobutyric acid (GABA), Glutamate (GLU) or serotonin (SER), which modulate these hormonal dynamics. Besides this neuronal control of the GnRH activity, the GnRH pulses remain under a crucial control of peptidergic innervation of Kisspeptin (Kiss-1) signaling. Kiss-1 acts as an upstream regulator of the HPO-axis and plays a key role in the onset of puberty and the sex steroid-dependent feedback regulation of GnRH neurons later in life [8]. So far, two major populations of Kiss-1 neurons have been identified and found to be regulated by sex hormones such as E2 in various ways. One region located at the arcuate nucleus (ARC) has been implicated in the negative sex steroid feedback process, while in the anteroventral periventricular nucleus (AVPV), KiSS-1 neurons were shown to be positively regulated by E2 stimulation [9].
In female rats, during the late afternoon proestrus, a surge of GnRH that is released from the neuronal terminals in the hypothalamus stimulates the anterior pituitary to release a timed bolus of LH and follicle stimulating hormone (FSH). Both gonadotropic hormones act on the ovary to induce ovulation and follicular recruitment (Fig.2A). Regulation of the preovulatory GnRH surge itself is primarily controlled by two types of input to GnRH neurons and their surrounding afferent neurons: E2 feedback from maturing ovarian follicles, and time-of-day output from the suprachiasmatic nucleus (SCN). Humans have a menstrual cycle of approximately four weeks consisting of a follicular phase followed by a luteal (progestational) phase. Briefly, before the onset of the mid-cycle LH surge, a small rise in plasma progesterone occurs, which is followed by the ovulatory LH surge. At this time, a rapid rise in plasma LH level takes place, which leads to the final maturation of the Graafian follicle and its rupture in about 16 to 24 hours after the LH peak. After the peak of the LH surge, the increase in FSH and E2 levels begins. The pre-ovulatory peak of E2 is reflected in an increased functional activity of the theca and granulosa cells, which leads to a rapid follicular maturation followed by ovulation. During the luteal phase, the follicular cells are luteinized and the post-ovulatory follicle becomes increasingly vascularized. LH is required for normal survival and function of the corpus luteum during the menstrual cycle. When the luteal peak declines, the menstrual phase sets in and the superficial layers of endometrial stroma begin to separate, which is accompanied by the loss of blood.

2. The role of estrogens and their receptors in reproductive axis

The female reproductive axis is a key target for estrogens, which play a major role in maintaining normal physiological function of fertility. During most of the normal female reproductive cycle, ovarian estrogens at low levels enforce a negative feedback upon the frequency of GnRH pulsatile release. Additionally, a positive feedback is confined to females during the pre-ovulatory period and occurs in response to sustained elevations of estrogens, which generate the induction of the pre-ovulatory surge of GnRH release [10; 11].

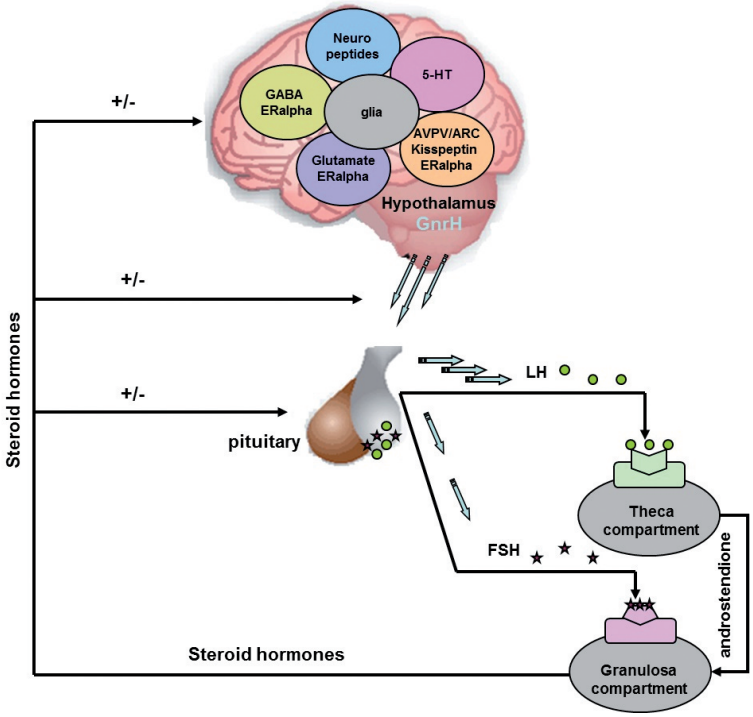

Fig. 1 Schematic model of Hypothalamus-Pituitary-Ovary (HPO) axis in humans. Upon pre-steroid hormonal stimulation, the hypothalamic GnRH is synthesized and released to stimulate LH and FSH secretion of anterior pituitary. These gonadotropins are then released and stimulate the steroid production synthesis of most developed ovarian follicle(s).

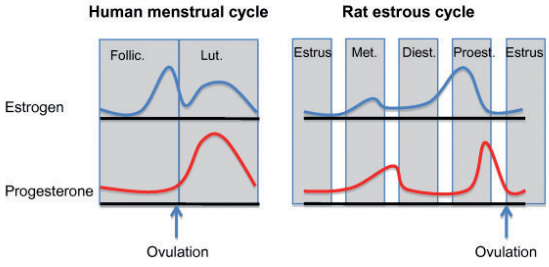

Fig. 2 Changes of hormone levels during human and rat female cycle period.
Source: http://www.biolmoodanxietydisord.com/content/figures/2045-5380-2-3-2.jpg

2.1 Classical and non-classical Estrogen receptors (ERs) signaling

The biological effects of E2 are classically mediated via their interaction with nuclear receptors that are members of a superfamily of ligand activated transcription factors. Estrogen receptor α (ERα) and estrogen receptor β (ERβ) are members of the nuclear receptor subfamily 3 (NR3A) and are encoded by two different genes [12; 13; 14]. The effects of estrogens can be mediated through several different pathways [15]. The classical mechanism of ER action involves E2 binding to ERs in the nucleus, induction of conformational change in the ERs that leads to dissociation of ER from chaperones, ER dimerization, and activation of the receptor transcriptional domain by binding specific, inverted palindromic sequences, the so-called estrogen response elements (ERE) in the promoters of target genes [16; Fig.3 A]. This genomic ERE-dependent signaling takes hours before the biological outcome manifests. Mounting evidence has emerged that ERs can regulate gene expression indirectly by modulating the function of different classes of transcription factors through protein-protein interactions in the nucleus via competition for transcription factors. Moreover, a rapid action of E2 has been more recently observed that was not mediated through gene activation and subsequent protein synthesis [17]. A distinct subpopulation of cell membrane-steroid hormone G protein-coupled receptors (GPR30) was cloned in the late 90s and first identified as an orphan member of the seven-transmembrane receptor family [18]. In early 2005, two reports provided evidence that GPR30 can bind E2 [19; 20]. The mechanisms of the activation of membrane ER by E2 occurs via Gβγ-subunit protein-dependent release of surface-associated HB-EGF and transactivation of the epidermal growth factor (EGF) receptor, triggering the activation of mitogen-activated protein kinases (MAPKs), extracellular-signal-regulated kinases 1 or 2 (Erk-1 and Erk-2, respectively) [21; 22].

2.2 ERs distribution in the HPO- axis

The ERs are diversely distributed throughout the reproductive axis and different ER subtypes were shown to exert various biological affects at the tissue level. ERα and ERβ are co-expressed abundantly in the reproductive tract of the human fetus [23]. Also, estrogen binding has been detected in the rat brain from embryonic day 21 (E21) [24] and in the mouse pituitary from embryonic day 17 (E17) [25].

Adult mammalian tissues are known to possess detectable levels of ERα, including the reproductive tracts, mammary glands, bone, the cardiovascular system, and regions of the brain [26]. ERβ mRNA is present abundantly in the epithelial cells of the prostate and ovary; moderately expressed in the testis, uterus, bladder, and lung; and low in the pituitary, thymus, and several other brain regions [27].

The sex steroids are important regulators of the neuronal control of reproduction in the hypothalamus. GnRH neurons themselves express ERβ, but the effects of estrogen on GnRH neurons are mostly mediated by a very complex network of neurons, such as glutamate and GABA neurons as well as glial cells expressing ERs [28]. Estrogens also modulate Kiss-1 neurons that are known to express ERs [29]. ERα and ERβ are also expressed in the membrane and cytoplasm of astrocytes. Estrogens play diverse functions in the pituitary and they are responsible for cellular proliferation and regulation of hormone synthesis, and its action is mediated through the ER. Both

ERα and ERβ are found in the pituitary of most species [30]. Several pituitary cells, such as lactotrophs and gonadotrophs, express ERα and are directly regulated by estrogen [31; 32; 33; 34]. Furthermore, a low expression of ERβ mRNA in the adult rat pituitary has been reported [35]. In the ovary, the main site of biosynthesis of estrogens in non-pregnant females, ERβ was shown to be a dominant ER subtype and its protein levels can vary during the estrous cycle in rats [36]. Also, mRNAs for ERα and ERβ are co-expressed in rat corpora luteum during pregnancy, and the expression of both mRNAs in luteal cells is up-regulated by prolactin and placental lactogens [37].

3. The role of Aryl hydrocarbon receptor (AhR) in reproductive axis

The aryl hydrocarbon receptor (AhR) and its nuclear partner Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) are abundantly present in the various mammalian ovarian cell types (oocytes, granulosa cells, and theca cells). The gene-depletion studies have implicated the AhR to fulfill an important role in controlling the development of the reproductive axis [38]. Indeed, AhR plays a crucial role in regulating the normal functioning of the organs comprising the female reproductive system from fetal development to adulthood [39].

3.1 AhR distribution in the HPO-axis

AhR is present in both male and female hypothalamus and shows a significant time-dependent increase in late gestational days [38]. The distribution of AhR gene expression in the brain follows the expression of the genes encoding glutamic acid decaroboxylase (GAD), which is the enzyme important in decarboxylation of GLU to GABA. GABA and glutamatergic neurons consist of unique ER and AhR- expressing neurons in the AVPV and mediobasal hypothalamus. Very little is known about the function role of the AhR in the pituitary gland development. Also, the role of AhR signaling in pituitary hormone synthesis and secretion is less understood. Some evidence suggests that AhR ligands can alter normal pituitary hormone production, and that increased AhR activity, likely though mutations in Aryl-hydrocarbon receptor (AhR) interacting protein (AIP) may lead to e.g. adenomas [50]. There is strong evidence to support a role in AhR influencing endocrine function, particularly with respect to gonad development and fertility [51, 52, 53]. In the developing ovary, AhR function is mainly implicated in ovarian follicle growth [46; 54; 55] and during postnatal life, AhR is involved in the ovulation onset, and steroidogenesis regulation [56]. AhR mRNA expression changes were also observed during the estrous cycle in rats [57].

3.2 AhR signaling

The AhR belongs to the basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) family of ligand-activated nuclear transcription factors. AhR regulates enzymes important to the metabolism of both endogenous substances (e.g., hormones) and exogenous

substances, involved in both the detoxification and bioactivation of xenobiotics [40]. In the absence of the ligand, an inactive form of AhR is found in the cytosol in a complex containing Hsp90 and p23 [41; 42]. Upon ligand binding, AhR undergoes a conformational change including dissociation from its cytosolic complex, migration into the nucleus, heterodimerization with ARNT and finally results in binding to DNA sequences known as dioxin response elements (DREs) and recruitment of coactivators, including SRC-1, SRC-2, and SRC-3 [41; 42; 43], to stimulate the transcription of target genes. Many (but not all) of the he target genes are involved in the regulation of phase I *Cyp1a1* and *Cyp1b1*, as well as phase II (UDP-glucuronosyl transferase and glutathione S-transferase) xenobiotic metabolism [44; 45; 46]. AhR has been also shown to regulate a wide spectrum of cellular processes including cell proliferation, differentiation, apoptosis, and intercellular communication [47; 48; 49].

3.3 AhR-ER cross-talk

AhR has been shown to affect E2 actions either by direct binding to ER and activation or inhibition of transcription of E2 downstream target genes in a ligand and concentration-dependent manner [58; 59]. AhR-induced impairment of ER activity may occur through a combination of several different mechanisms [60]. AhR may affect ER mediated transcription through the direct inhibition by the activated AhR/ARNT heterodimer via binding to inhibitory XRE (iXRE) present in ER target genes (Fig.3). AhR activation may also impair ER mediated transcriptional activation by sequestering of shared coactivators. The competition for cofactors depends on the amounts and types of cofactors expressed in the cell, which are highly dependent on cell and tissue type [59]. Interestingly, it has been shown that ARNT can also act as a co-activator of ERα and ERβ [61]. Next, the increase of proteasome-dependent degradation of ERα through the activated AhR results in ubiquitination of AhR and ERα and degradation of both proteins [62]. Finally, the enhancement of the expression of AhR targeted CYP1A1 and CYP1B1 genes leads to an increase of metabolism of estrogen synthesis/metabolism [63] (Fig. 3).

4. Endocrine disrupting compounds (EDCs) and reproductive axis

4.1 Endocrine disruption, definition

In the past few decades scientific concern has been raised regarding some man-made chemicals, such as phthalates and polychlorinated biphenyls that were shown to affect the reproductive tract in humans, wildlife or aquatic organisms by disrupting their endocrine functions [64; 65; 66]. Various definitions have been used to define endocrine disruptor compounds (EDCs) by European Union (EU), the World Health Organization (WHO) and the US Environmental Protection Agency (EPA). WHO accepted the EDC definition in in a broader sense as "*an exogenous substance or mixture that alters function(s) of the endocrine system and consequently produces adverse health effects in an intact organism or its progeny or (sub)population*". EPA implemented the Kavlock et al. (1996) [66] more detailed of EDCs as: "*an*

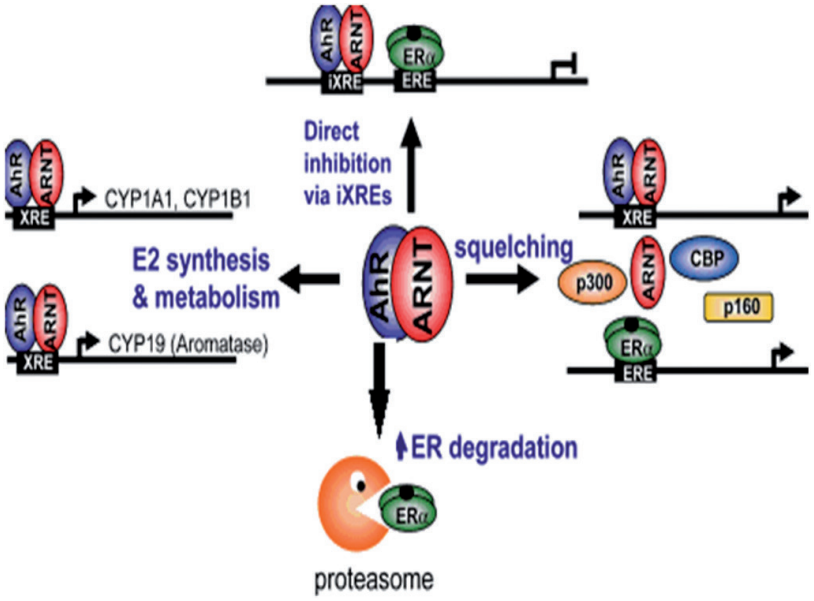

Fig. 3 Proposed mechanisms of crosstalk between AhR and ER signaling pathways. Adopted by Matthews and Gustafsson, 2006 [60] with further modifications.

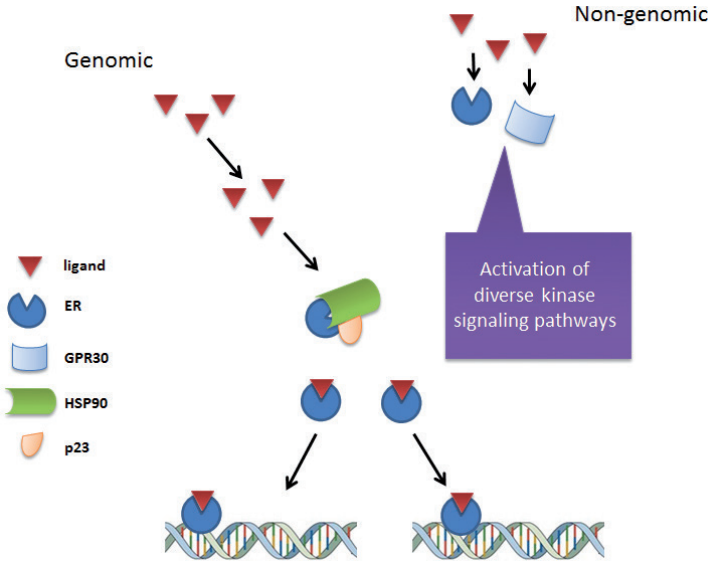

Fig. 4 Genomic and non-genomic action of EDCs.

exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance or homeostasis, reproduction, development and/or behavior" with further modifications starting as: "*...does not consider endocrine disruption to be an adverse effect per se, but rather to be a mode or mechanism of action potentially leading to other outcomes, for example carcinogenic, reproductive, or developmental effects, routinely considered in reaching regulatory decisions. Evidence of endocrine disruption alone can influence priority setting for further testing and the assessment of the results of this testing could lead to regulatory action if adverse effect are shown to occur.*" (http://www.epa.gov/endo/pubs/edsparchive/2-3attac.htm). Finally, under the EU legislation for chemicals "Registration, Evaluation, Authorisation and Restriction of Chemicals" (REACH), potential endocrine disruption is considered as a crucial element to identify substances of high concern (EU, 2006). In 1996, the European Commission implemented a policy of regulation with regard to suspected EDCs. As a result, a Community strategy for endocrine disrupters was adopted including short-, medium- and long-term actions aiming at the establishment of the priority list of EDCs for the further evaluation of their role in endocrine modulation. Endocrine disruption is a serious public health problem. Thus, an appropriate authorization of potential EDCs and risk assessment of these chemicals is necessary for the authorization of their use. Without doubt, deleterious effects on human health and on the environment should be prevented whenever possible.

4.2 EDCs and the reproductive system

Endocrine disruptors can be classified into two groups: natural substances, such as endogenous hormones of animal origin or phytoestrogens, and man-made industrial chemicals. The major source of exposure to EDCs is via food. After uptake, EDCs may exert a diverse spectrum of adverse effects on the female reproductive system via disruption of the feedback loops of the hormonal and homeostatic systems in the body. The alterations in the endocrine system among the hypothalamic, pituitary, and ovarian components of the reproductive axis can have a marked input on female cycle and additional consequences on sexual development and functioning [67; 68] Also, consistent detection of EDCs in human samples such as blood plasma or follicular fluid has raised concern that exposure to EDCs may have an effect on human fertility [69]. The EDC- induced perturbations of HPO-axis may include changes in Kiss-1 and GnRH neuropeptide expression [70; 71] or impaired fecundity through the blockage of the ovulation [72] and changes in ovarian follicle development through the meiotic disruption [73]. As a consequence, the oocyte competence is diminished which may result in perturbation of embryonic development [74; 75]. The hormonal imbalance caused by EDCs may also lead to serious clinical disorders such as endometriosis, disorders of the uterus and ovary, e.g. premature ovarian failure (POF) and polycystic ovary syndrome (PCOS), infertility or increased prevalence of some tumors including breast cancer [76; 77; 78; 79; 80].

4.3 Mechanisms of action of EDCs

The adverse effects of EDCs on endocrine system are exerted mostly due to the estrogenic and/or anti-estrogenic nature of these compounds [81]. The HPO-axis provides several potential targets for modes of interaction for EDCs. Some EDCs were shown to act at receptor level, including the nuclear receptors such as ERs, androgen receptors (ARs), mineralocorticoid receptors (MRs), glucocorticoid receptors (GRs), progesterone receptors (PRs), thyroid receptors (TRs) and peroxisome proliferator-activated receptors (PPARs). Steroid hormone synthesis has also been recognized as an important target of EDCs [82]. The hormone metabolizing enzymes, including aromatase (CYP19A1) [82] 5α-reductase [83], 3β-hydroxysteroid dehydrogenase (3β-HSD) [84], 17β-hydroxysteroid dehydrogenases (17β-HSDs) [85] and 11β-hydroxysteroid dehydrogenases (11β-HSDs) [86] were already shown to be affected by EDCs. In addition, EDC effects can be mediated via alteration of the biosynthesis and metabolism of endogenous hormones levels, which is manifested through perturbation of their release, clearance and homeostasis [87; 88].

Similarly to E2, EDCs that target ER signaling can modify genomic and non-genomic ER activity through direct interactions with ERs or indirectly through other transcription factors such as the AhR or modulation of enzymes critical for normal estrogen synthesis and metabolism (Fig.4). In this thesis the main emphasis was put on chlorinated dioxin-like compounds and plant-derived phytoestrogens with a further description of the mechanisms of their action.

4.4. Dioxin disruption of female reproduction via Aryl hydrocarbon Receptor (AhR)

Chlorinated dibenzo-p-dioxins (PCDDs) and related compounds represent a family of ubiquitous environmental pollutants, which are unintentionally released into the environment through various industrial processes, such as paper bleaching, drinking-water disinfection, and incineration of waste [89]. Among PCDDs, the model compound of this class of chemicals is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). According to the Agency for Toxic Substances and Disease Registry, TCDD is the most toxic dioxin and it has been designated by International Agency for Research on Cancer (IARC) as a human carcinogen [89]. TCDD has been linked with many adverse reproductive outcomes in rodent studies, including fetal loss and birth defects [89]. Exposure to humans occurs primarily through consumption of contaminated foods, particularly meat, dairy and fish [89]. TCDD has been shown to decrease sex steroid hormone synthesis, but at the same time also increase catabolism of E2, the key hormone of ovarian steroidogenesis [90]. Furthermore, the anti-estrogenic properties of TCDD on ER signaling are well documented [59]. Nonetheless, some dioxin-like compounds and other AhR ligands can also exhibit weak estrogenic activity [58]. It is generally accepted that toxic effects by TCDD and related compounds are predominantly mediated by AhR [41; 42; 43].

Dioxin-induced reproductive impairment may occur at any or all of three levels of the HPG axis during postnatal life. *In vitro* cell culture studies demonstrated disruptive effects of TCDD and PCBs at neurotransmitter levels and an increase of GnRH mRNA levels [71]. At the pituitary level, exposure to TCDD and PCBs directly attenuated

basal LH and FSH secretion and altered pituitary responsiveness to GnRH in female rats [91; 92]. With respect to ovary, some *in vivo* and *in vitro* studies have indicated endocrine disrupting effects of dioxins at gonadal level at least in part by their direct action on this organ [91; 93; 94]. Ovarian Aromatase (CYP19A1) is the crucial enzyme that is responsible for catalyzing the final conversion of androstenedione and/or testosterone (T) to estrone (E1) and E2, respectively [95]. TCDD was shown to exert diverse effects on aromatase (CYP19A1), by decreasing its mRNA and protein content in JEG-3 cells [96] and granulosa cells [97; 98]. In contrast, others have observed that TCDD induces CYP19 expression and activity in primary Sertoli cells [99] and human placental cells [100].

4.5 Phytoestrogens as EDCs

Phytoestrogens are polyphenolic, non-steroidal compounds that are either of plant origin or metabolic derivatives of precursors present in plants eaten by humans [101]. The exposure to phytoestrogens occurs primarily through dietary intake of beverages and foods containing fruit, herbs, and vegetables. Most of phytoestrogens are absorbed as their glycoside conjugates containing glucose or carbohydrate moieties [102; 103]. Bioavailability of phytoestrogens varies among individuals and depends on many factors, such as habitual diet, gender, different individual metabolism patterns that might be determined by genetic factors and different bacterial flora. After a hydrolysis step by gut microflora enzymes, phytoestrogens are transformed into aglycones, which allows a more efficient passing through the intestinal digestive barrier. Phytoestrogens bear a structural similarity to E2. They have been classified as natural selective estrogen receptor modulators (SERMs) and can have both estrogenic and anti-estrogenic effects depending on the concentrations of circulating endogenous estrogens and estrogen receptors [101; 104]. In addition, the estrogenic potency of phytoestrogens varies within the particular phytoestrogen group.

The principal phytoestrogen classes are flavonoids, lignans, coumestans and stilbenes [105; 106; 107]. Flavonoids are an ubiquitous class of phytoestrogens found in dietary sources and further sub-classified as chalcones, flavones, flavonols, flavanones, flavanols, anthocyanins, and isoflavones. Soy foods in particular are rich in isoflavones, and Asian populations habitually consume large amounts of these foods. Lignans are more widespread and occur more frequently in Western diets than isoflavones. The usual intake of phytoestrogens is higher in Asia (400 mg/day) and by vegetarians (345 mg/day) than Western Europe (80 mg/day) [108]. Many animal studies also indicate that phytoestrogens can compete for ER binding and modulate normal ER action in target tissues. In transgenic estrogen reporter mice, genistein treatment inhibited the hepatic estrogenic response by E2, indicating that phytoestrogens can modify the effects of endogenous estrogens [109]. Moreover, phytoestrogens can also affect the estrogen biosynthesis and menstrual cycle [110; 1011]. Also, decreased urinary excretion of E2, estrone, estriol, and total estrogens has been reported in pre-menopausal women consuming isoflavone-supplemented diets for three menstrual cycles [112].

The decrease in circulating estrogen concentrations after phytoestrogen intake may be a result of interference with estrogen biosynthetic enzymes such CYP19 and HSD

[113]. HSD catalyzes the inter-conversion of the relatively inactive 17β-keto steroids, such as estrone and androstenedione, to active 17β-hydroxyl steroids such as E2 and T [114]. In the breast tissue of postmenopausal women, HSD and CYP19 are responsible for the local production of estrogens, and overexpression or increased activity of these enzymes is associated with breast cancer [115; 116].

5. Need for non-animal alternatives

The use of animals is still compulsory for hazard and risk assessment of chemicals, plant protection products, biocides, feed additives and pharmaceuticals. However, the awareness of the need for alternative methods to replace, refine or reduce animal testing in the light of 3R principles of animal tests by Russell and Burch [117], has increased dramatically. High priority has been put to develop new strategies for non-animal tests particularly directed to reproductive and developmental toxicology, because large numbers of animals are still annually used in these particular fields of toxicology [118; 119]. The complexity of the reproductive cycle in mammals is one of the major obstacles why success in non-animal methods for these fields has made limited progress up to now. Thus, the assessment of reproductive and developmental toxicity is still based on *in vivo* animal test systems in spite of societal demands for reduction of the number of test animals. These tests are still complex by nature and encompas the entire reproductive cycle from gametogenesis through embryonic and postnatal development to adulthood. In addition, these *in vivo* test are cost and time consuming. As estimated by van der Jagt et al. (2004) almost four million of animals might be used under REACH, with 70% used to assess reproductive and developmental toxicity [118].

Among non-animal test systems, *in vitro* cell cultures represent valuable tools to elucidate mechanistic aspects. Unfortunately, the application of these tests for regulatory purposes is still very limited as no corresponding guideline is officially in place. There is a wide range of alternative cellular test systems available, with the majority being derived from relevant organs and tissues that are frequently derived from human origin or embryonic life stages. With regard to the testing of EDCs, the Organisation for Economic Co-operation and Development (OECD) has published a Conceptual Framework (CF) with a detailed description of the mainly *in vivo* test assays that have been validated and adopted (OECD, 2012). In a similar approach, the European Chemical Agency (ECHA) developed guidance to evaluate the information related to endocrine activity in aquatic toxicity testing for REACH. Nevertheless, *in vitro* modeling of the full reproductive cycle to predict human reproductive and developmental risk remains a monumental challenge. Still, a journey of a thousand miles begins with a single step.

Scope of the thesis

The aim of this thesis was to study alterations of EDCs in (part of) the HPO-axis using available *in vitro* cell models. The main focus was on mechanisms of action of EDCs upon neuroendocrine and steroid hormone modulations. To this purpose, several cells of hypothalamic, pituitary or ovary origin were used and evaluated for their value in determining EDC- responsive biomarkers of HPO-axis. To achieve this purpose, molecular indicators of endocrine modulation at the level of the hypothalamus, pituitary and ovary level were studied. As a result, this PhD thesis provides some interesting cell models, which could support future identification and elucidation of the modes and mechanisms of action of EDCs within the HPO-axis.
The book contains chapters as follows:

Chapter 1. General Introduction

Chapter 2. Rat Primary cultures dioxins, dioxin like compounds

In this chapter, we introduced primary tissue cultures of hypothalamus, pituitary and ovarian granulosa-theca (HPO) cells, which were derived directly from healthy female rat tissues. The action of two dioxin-like compounds (TCDD and PCB126) and one non-dioxin-like PCB (PCB153) on steroid hormones and their receptors was investigated in the three different HPO compartments.

Chapter 3. Description of the novel rat hypothalamic cell line GnV-3 and the role of AhR at hypothalamus level

This chapter describes a potential novel *in vitro* model for the hypothalamus. Currently, a suitable *in vitro* model to study potential effects of exogenous compounds at the cellular and molecular level in the hypothalamus is lacking. A suitable hypothalamic model or cell line should ideally expresses the aryl hydrocarbon receptor (AhR). Here, we investigate the applicability of the AhR-expressing rat hypothalamic GnV-3 cell line using 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as a model compound. In addition, neuroendocrine effects of AhR activation and the involvement in modulation of GnRH pulsality and expression of neurotransmitters as well as in food intake were determined on a cellular and molecular level.

Chapter 4. Human granulosa-derived tumor KGN cells

This chapter describes the effects of different classes of phytoestrogens: Naringenin, 8-prenylnaringnein, genistein, coumestrol, quercetin and resveratrol on ovarian steroidogenic enzymes such as steroidogenic acute regulatory protein (*StAR*) and *CYP19A1* and its specific promoter-expression in the human granulosa-tumor derived cell line KGN. Furthermore, the phytoestrogens were tested as possible modulators of KGN tumor cell behavior.

Chapter 5. Porcine oocytes – reproductive model
In this chapter we aimed at investigation of possible disrupting effects of phytoestrogens: naringenin (NAR) and 8- prenylnaringein (8-PN) on the developmental competence of oocytes using an *in vitro* porcine oocyte model. The main focus was put on the maturation stages i.e cumulus expansion and nuclear maturation. Furthermore, we tested to which extent the competency of the oocyte to develop into blastocyst stage can be disrupted by phytoestrogens by applying the embryo cultures.

Chapter 6. General discussion
This chapter summarizes the research on *in vitro* HPO-axis models described in this thesis. Additionally, the value of co-culture testing and the use of *in silico* models HPO is discussed. The *in vitro* toxicity testing can be more valuable compared to whole organism testing as these assays provide tools to investigate chemical-specific molecular/cellular perturbation(s) and characterize its dose-dependent effects on set endpoints.

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CHAPTER 2

The effects of 2,3,7,8-tetrachlorodibenzop-dioxin (TCDD) 3,3′,4,4′,5 pentachlorobiphenyl (PCB 126) and 2, 4,5,2′,4′,5′,-hexachlorobiphenyl(PCB153) on hypothalamus-pituitaryovary (HPO)-axis in rat primary cultures.

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Abstract

Dioxins and polychlorinated biphenyls (PCBs) may exert a variety of adverse effects on the reproductive system. In this study, TCDD (10 nM) and dioxin-like PCB126 (100 nM) were shown to act as endocrine disruptive compounds (EDCs) on the hypothalamus-pituitary-ovary (HPO) reproductive axis, mainly through the activation of aryl hydrocarbon receptor (AhR) signaling. We showed that, in all HPO-axis compartments, TCDD and PCB126 elevated their sensitive markers *CYP1a1* and *AhRR mRNA* after 24 hours. No changes in Gonadotropin-releasing hormone (*Gnrh1*) mRNA were detected, however the *Kiss-1* mRNA levels were significantly up-regulated up to 2 fold for all tested congeners. More pronounced effects of dioxin action were seen at pituitary and ovary levels. Both TCDD and PCB126 increased the mRNA levels of *Fshbeta* up to 1.4 and 1.7 fold, respectively, wherein the *Fshr* mRNA were significantly down-regulated. TCDD and PCB126 exerted their anti-estrogenic effects down-regulating the *Cyp17* and *Cyp191a1* mRNA however no changes in E2 protein levels were be observed after 24 and 72 hours. PCB153 did not exert pronounced effects on all HPO compartments. This study presents the first step in characterization of dioxin-like effects upon HPO-axis *in vitro.* The results support *in vivo* animal data on dioxin-like compounds in reproductive system and provide additional information with respect to mechanistic action of these toxic compounds on HPO system.

1. Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) and polychlorinated biphenyls (PCBs) belong to a group of wide-spread persistent organic pollutants present ubiquitously in the environment. As a result of their lipophilic nature and metabolic stability they can accumulate in the food chain building up in the fat tissue content of animals [1; 2]. Human exposure to dioxins and dioxin-like compounds occurs mainly through the diet. Dioxins can pass the placenta (IARC, 1997) and are present in human breast milk [3]. Among dioxins, 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) is considered to be the most potent environmental toxicant [4]. In experimental animals, TCDD has been shown to cause cancer in a dose-dependent manner [5]. With respect to the reproductive system, dioxins have been shown to be responsible for a variety of adverse effects on sexual development and physiology of the reproductive system [6]. In experimental animals, the endocrine system has been shown to be one of the most sensitive for TCDD and PCBs [7]. A compelling body of evidence indicates that the biochemical and toxicological responses to TCDD have a similar mechanism of action in both animals and humans. TCDD and dioxin-like PCBs can act as endocrine disruptive compounds (EDCs) on the hypothalamus-pituitary-ovary (HPO) axis via the activation of Aryl hydrocarbon Receptor (AhR) signaling pathway, contrary to non-dioxin like PCBs which have little affinity or are AhR-inactive and display a different toxicological profile.

The AhR is a member of super family of basic (bHLH)-Per-ARNT-Sim (PAS) transcription factors [8]. Although initially considered a xenobiotic sensor, there is now a vast body of evidence supporting the role of AhR in many physiological processes including in the development and maintenance of the female reproduction system. AhR-knockout mouse models show a variety of reproductive deficiencies including small litter size at birth, poor survival of pups during the first two weeks after birth, and increased mortality of offspring after weaning [9]. With respect to the hypothalamus-pituitary-ovary (HPO) axis, AhR function is predominantly implicated in ovarian follicle growth, ovulation onset, and steroidogenesis [10]. Moreover, AhR mRNA expression changes are observed during the estrous cycle at ovarian level [11]. An important mode of action herein appears to arise from the inhibitory crosstalk between the AhR complex and Estrogen Receptor (ER) signaling [12]. The molecular basis of AhR-ER interaction includes proteasome-ER dependent degradation [13], rapid metabolism of E2 via AhR-driven genes [14], direct inhibition through inhibitory xenobiotic response elements (XREs) located in estrogen (E2)-responsive gene promoters [15; 16] and squelching of common cofactors [16;17;18].

Many animal experimental studies report the female reprotoxic effects of dioxins. In a three generation reproductive toxicity study, exposure to TCDD decreased the fertility rate and gestational and neonatal survival [19]. Also, a significant loss in weight of the reproductive organs including ovarian masses have been demonstrated after exposure to PCB 77 in mice encompassing the reduction in the number of foetal germ cells in two mouse strains [20]. Oral administration of PCB i.e. Aroclor-1248 to females was linked to irregular menstrual cycles in rhesus monkeys [21], reduction of ovulation occurrence [22; 23] or even loss of ovarian cyclicity in adult rats [24].

Moreover, upon fertilization, the number of implantation sites, embryo toxicity and litter size were diminished after dioxin treatment [20; 25; 26].
Epidemiological studies of TCDD and dioxin-like compounds are limited and their impact on human health is inconsistent. Most of the human data are based on occupational exposure or environmental industrial incidents. One major incident was the one in Seveso in 1976, where an explosion in a 2,4,5-trichlorophenol (TCP) production plant led to emission of a 'toxic cloud' including TCDD. The exposed population showed increased all-cancer mortality 15–20 years after exposure among those living in the most contaminated area. No known increase in the number of birth defects attributed to dioxin exposure was observed. However, in the area with the highest levels of contamination an unusual sex ratio (2:1 females /males) among the children born between 1984 and 1987 was observed. However 10-fold increase in TCDD exposure of pre-menarcheal Seveso women was associated with increase of 0.93 days in menstrual cycle length [27].
Dioxin-induced reproductive impairment may occur at any or all of the three levels of the HPO-axis. *In vitro* and *in vivo* studies demonstrated disruptive effects of TCDD and a mixture of PCBs at neurotransmitter levels [28; 29; 30] and increase of synthesis of gonadotropin releasing hormone (GnRH) mRNA levels [31;32]. At pituitary level, exposure to TCDD and PCBs directly attenuated basal LH and FSH secretion and altered pituitary responsiveness to GnRH in female rats [33; 34]. In the ovary, dioxins were shown to act directly via steroidogenic enzyme attenuation or indirectly via hypothalamus-pituitary feedback through the inhibitory gonadotropin action [24]. In human luteinizing granulosa cells, TCDD decreased estradiol [35], but did not alter progesterone production [36]. *In vitro* results indicated disruptive effects of TCDD upon steroidogenesis by inhibiting the mobilization of cholesterol from outer mitochondrial membranes [37] presumably via steroidogenic acute regulatory (StAR) protein in male rats. Also modulation of mRNA transcription of P450 enzymes involved in the steroidogenic cascade such CYP11a1 or CYP19 were reduced in *in vitro* cultured prepubertal (PP) and adult (A) rat granulosa cells (GC) with consequent reduction of estrogen secretion at 48 hours [38]. Moreover, the potency of TCDD is dependent upon species, strain, developmental stage, gender, and tissue type [39].
Yet, due to the great complexity of the female reproductive axis with its highly multiplex and integrated signal transduction pathways, understanding of the molecular endpoints affected by TCDD and its related compounds remains poor. Primary tissue-derived cell cultures from healthy animal tissues can provide physiologically relevant useful models for toxicology research and mechanistic understanding of effects. For example, ovarian primary cultures have widely been used as helpful model systems to study the complex nature of granulosa cells [40]. The present study was designed to explore the endocrine disrupting properties of TCDD, PCB126 and PCB153 on female adult rat HPO -axis *in vitro* by employing primary hypothalamic, pituitary and ovary cultures. These data will aid to further elucidate key hormonal regulators of HPO-axis and might be used to study the mechanisms of action of EDCs on the HPO-axis.

2. Material and methods

2.1 Chemicals and reagents

2,3,7,8-tetrachloro-p-dibenzodioxin (TCDD), 3,3',4,4',5-Pentachlorobiphenyl (***PCB 126***), 2,2',4,4',5,5'- hexachlorobiphenyl (PCB 153) were purchased form Wellington Laboratories Inc. (Guelph, Ontario, Canada) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Fluka Chemie). Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH) were purchased from Sigma (Fulka, Chemie) and dissolved in PBS.

2.2 Animals

All experiments were conducted in accordance with Dutch law and are approved by the Ethical Committee for Animal Experiments of Utrecht University. The animal study was performed as part of EU-funded project SYSTEQ (FP7-ENV-226694) [41]. Female Sprague-Dawley rats were purchased at 9 weeks of age from Harlan laboratories (Venray, The Netherlands) and allowed to acclimatize for 1 week. The rats were housed in standard cages (46x35x19cm) and conditions (temperature 23 ± 2°C, 50% to 60% relative humidity, 12 hours dark and light cycle) with free access to food and water. Rats were sacrificed by CO_2/O_2 in the morning at 8:30-9:00. In this study, the hypothalami, pituitaries and ovaria of non-ovulating female rats (diestrus I and early stage of diestrus II) were used, as assessed by the EC40 Estrus Cycle Monitor (Fine Science Tools, Foster City, CA, USA) which principle lays on electrical impendence of epithelial cell layer of vaginal mucosa. After vaginal measurement, the organs were immediately harvested under sterile conditions and used immediately for primary cultures.

2.2.1 The hypothalamus *in vitro* culture

For primary adult rat hypothalamic culture, six healthy unexposed Female Sprague-Dawley rats were used. Upon dissection, whole hypothalami were immediately placed in cold PBS supplemented with glucose and antibiotics solution. The neurons were isolated using Papain digestion system [42] with some further modifications. Briefly, six hypothalami were pulled, minced into small pieces and incubated with papain (24U/mL) for 15 minutes in 37°C. Digestive tissue was then spinned and flushed 20-30 times by up and down pipetting. The cells were plated into 5 µg/ml poly-D-lysine-coated (Sigma, Fluka Chemie) 12-well plates in medium containing Neurobasal medium whithout phenol red (Invitrogen-Gibco) supplemented with B27 supplement (Invitrogen-Gibco), 1% charcoal-stripped FBS (Hyclone) (Invitrogen), 200 mM GlutaMax (Invitrogen), 25 µM glutamate (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 ng/ml bFGF (Invitrogen). Neuronal cells were cultured for eight days. Each day, the debris from the culture was gently removed using a pipette. The cells were cultured at 37°C in a humidified atmosphere at 5% CO_2.

2.2.2 The whole pituitary *in vitro* culture

Dispersed pituitary cells from whole pituitary glands were prepared as described by Zhou et al. (1998) with some minor modifications [43]. Briefly, whole pituitaries

from six rats were cut into small pieces and incubated in enzyme mixture containing: 0.5% collagenase type I and 5µg/mL DNase I in Hank's solution for 30 and 5 minutes, respectively. After enzymatic digestion cells, were washed with 0,03% EDTA- PBS and then incubated with 0.05% trypsin for 20 minutes. Nylon70µM filter was used to strain cell suspension, which were then seeded in the poly-d-lysine coated 12-well plates in DMEM-F12 (1:1) without phenol red medium supplemented with 10% FBS (Hyclone) 100 U/ml penicillin and 100 ug/ml streptomycin (Invitrogen). Pituitary cells were cultured for 48 hours. After that time, the cells were used for an experiment. The cells were cultured at 37°C in a humidified atmosphere at 5% CO_2.

2.2.3 The whole ovary *in vitro* culture

Culturing of ovarian tissue *in vitro* was performed as described in Magoffin et al. (1988) with some minor modifications [44]. For primary adult rat ovaria culture, whole ovaria from six healthy Female Sprague-Dawley rats were harvested under sterile conditions and placed in cold medium 199 (Invitrogen). Theca and granulosa cells were isolated using collagenase-DNAse solution. Briefly, twelve whole ovaria were pulled, minced into small pieces and incubated with 4mg/mL collagenase-10µg/mL DNAse solution for 90 minutes in 37°C. Digested tissue was flushed 20-30 times by up- and down with successively decreasing pipette diameter. The granulosa and theca (ovarian) cells were plated into 12-well plates (5 x 10^5 cells/well) in DMEM-F12 (1:1) without phenol red medium supplemented with 10% FBS (Hyclone) 100 U/ml penicillin and 100 ug/ml streptomycin (Invitrogen). Ovarian cells were cultured for 48 hours before exposure. The cells were cultured at 37°C in a humidified atmosphere at 5% CO_2.

2.3 Cell viability (Alamar Blue (AB))

The pituitary and ovary cells (approximately 0.5 x 105) were seeded onto 96-well plates. Neurons isolated from hypothalamus were seeded onto 5 µg/ml poly-D-lysine-coated 24-well plates in phenol red-free Neurobasal differentiation medium. Briefly, all culture cells were exposed to TCDD (10 nM), PCB126 (100 nM) and PCB153 (10 µM) for 24 hours. The AB (Invitrogen, Basel, Switzerland) assay was performed after a 3- and 2 hours incubation for neuronal and pituitary/gonadal cells, respectively at 37°C with AB solution (1:20). The redox reaction in which AB was reduced by cells was measured by absorbance readings at 540 and 595 nm.

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

Total RNA was isolated from the neuronal, pituitary and ovarian cells by phenol–chloroform extraction using RNA Instapure kit (Eurogentec, Liege, Belgium). RNA's purity and concentration were determined spectrophotometrically at an absorbance wavelength of 260/280nm and 230/260 nm, respectively. For each experiment, 40 ug/mL of total RNA was used. Complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis Kit, according to the manufacturer's instructions (Biorad, Veenendaal, The Netherlands). The obtained cDNA was diluted 10 times and stored at -20 °C until further analysis. The primers for rat *AhRr, Cyp1a1, Kiss-1, Lhbeta, Fshbeta, GnRHreceptor, Cyp19a1, Cyp17, Lhr, Fshr* and *Actin beta* were designed

on National Center for Biotechnology Information (NCBI) and checked with Blast (nucleotide nonredundant database) to confirm specificity. The primers for *Gnrh1* has been described previously [45]. The primer pairs used in the qRT-PCRs are listed in Supplementary Table 1.

2.5 Rat Luteinizing hormone (rLH) and Rat follicle stimulating hormone (rFSH) RIA Ki

After each experiment, the media of primary pituitary cultures were immediately collected and stored at -80°C before use. The rat LH and FSH RIA Kits were purchased from the Institute of Isotopes (Budapest, Hungary). LH and FSH measurements were conducted according to the manufacturer's instruction.

2.6 Rat Estradiol ELISA Kit

After each experiment, the media of primary ovarian cultures were immediately collected and stored at -80°C before use. Rat estradiol kit was purchased from GENTAUR Europe (Eersel, the Netherlands). The estradiol measurement was conducted according to the manufacturer's instruction.

2.7 Data analysis and statistics

Experiments were performed in three independent experiments that were performed in triplicate, unless stated otherwise. Statistical significance in difference of the means was calculated using un-paired Student's t-test using Prism 6.0 (Graph Pad Software, San Diego, CA, USA).

2. Results and discussion

The toxic effects of dioxin and dioxin-like compounds are known to be organ-, cell-, and species-specific [46]. The present study was designed to explore to which extent dioxin, dioxin-like and non-dioxin-like compounds exert their endocrine disrupting properties on the female rat HPO-axis by employing *in vitro* hypothalamus, pituitary and ovary primary cultures (Fig.1) and investigating the mechanisms of the action of TCDD and PCB126 and PCB 153 on the main components of this reproductive axis.

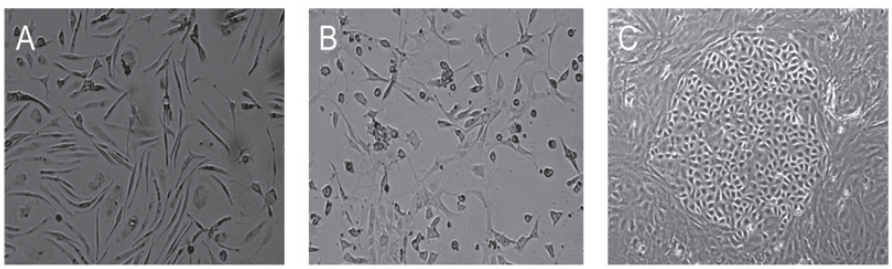

Fig.1 Primary culture of female rat hypothalamus (A); pituitary (B); ovary (C). Magnifications 20X. Details on isolation and culture are provided in Materials and Methods section.

2.1 TCDD and PCB 126 elevate Cyp1a1/AhRR mRNA levels in primary hypothalamus- pituitary – ovary cultures

The *Cyp1a1* gene encodes the enzyme cytochrome P4501A1, which oxygenates various xenobiotics as part of their stepwise biotransformation [47]. *Cyp1a1* expression and/or activity are generally used as positive control for AhR activation. As shown in Figure 2, TCDD and PCB126 increased *Cyp1a1* mRNA levels in hypothalamus, pituitary and ovarian cultures, confirming the presence of an active AhR in these cells. TCDD (10 nM) was the most potent inducer of *Cyp1a1* mRNA and elevated its levels up to 8-, 42- and 472 fold in hypothalamus, pituitary ovary cultures, respectively (Fig.2). PCB126 is considered to be 10-fold less potent than TCDD and consequently is assigned a toxic equivalent value (TEF) of 0.1 [48]. Here, 100 nM PCB126 indeed increased *Cyp1a1* mRNA levels up to 6.5-, 32- and 193 folds in hypothalamus, pituitary and ovary cultures, respectively, comparable to 10 nM TCDD (Fig.2). It is well-known, that both TCDD and the dioxin-like congener PCB126 exerts adverse effects in the body predominately via triggering AhR signaling [49]. The non-dioxin-like PCB153 (10 µM) did not increase *Cyp1a1* mRNA levels in pituitary and ovarian cultures, as expected. Interestingly, *Cyp1a1* mRNA was significantly decreased ($p<0.001$) to 44% of vehicle control-treated cultures after exposure to PCB153 in the hypothalamus cultures. The AB cell viability test did not reveal any cytotoxicity after exposure to tested congeners for 24 hours (data not shown). A similar trend as for *Cyp1a1* mRNA levels was determined with respect to the induction of Aryl hydrocarbon receptor repressor (*AhRR*) mRNA expression by all tested compounds for each compartment of HPG axis (Fig.2). It has been recognized that the toxicity exerted by PCB153 does not require AhR activation. However, an unproductive binding of PCB 153 to the rat AhR has been demonstrated [50]. It remains obscure why AhR-PCB153 complex did not bind to a XRE sequence, resulting in sequestration of the AhR. The non-coplanar PCBs were suggested to act as ligands for the constitutive androstane receptor (CAR) and/or the pregnane-X receptor (PXR) [51], and may thus activate CAR/PXR target genes expression. Indeed, at high doses, PCB 153 (1-10 µM) has been shown to activate CAR in human hepatocellular carcinoma Huh7 cells [52] and induce CAR-mediated CYP2B1/2 metabolizing enzymes [53; 54; 55]. Patel et al (2007) demonstrated a link between AhR and CAR and the ability of activated AhR to increase CAR activity. Interaction or competition between the AhR and CAR receptor pathways needs further testing [56].

2.2 Effects of dioxins at hypothalamus level

The hypothalamus plays a key role in controlling reproduction by the coordination of hormonal signaling pathways crucial for maintenance of fertility and reproduction. In essence, the GnRH neurons are the principal neurons responsible for the final output signal of the hypothalamus. To determine the direct effects of dioxins on hypothalamic *Gnrh1* and kisspeptin (Kiss-1) gene expression, the hypothalamic neurons were exposed *in vitro* to TCDD (10 nM), PCB126 (100 nM) and PCB153 (10 µM) for 24 hours. As shown in Fig.3, all tested congeners did not attenuate the mRNA expression of *Gnrh1*. However the *Kiss-1* mRNA levels were statistically significantly up-regulated up to 1.9, 2 and 2.1-fold compared with vehicle-treated control cells

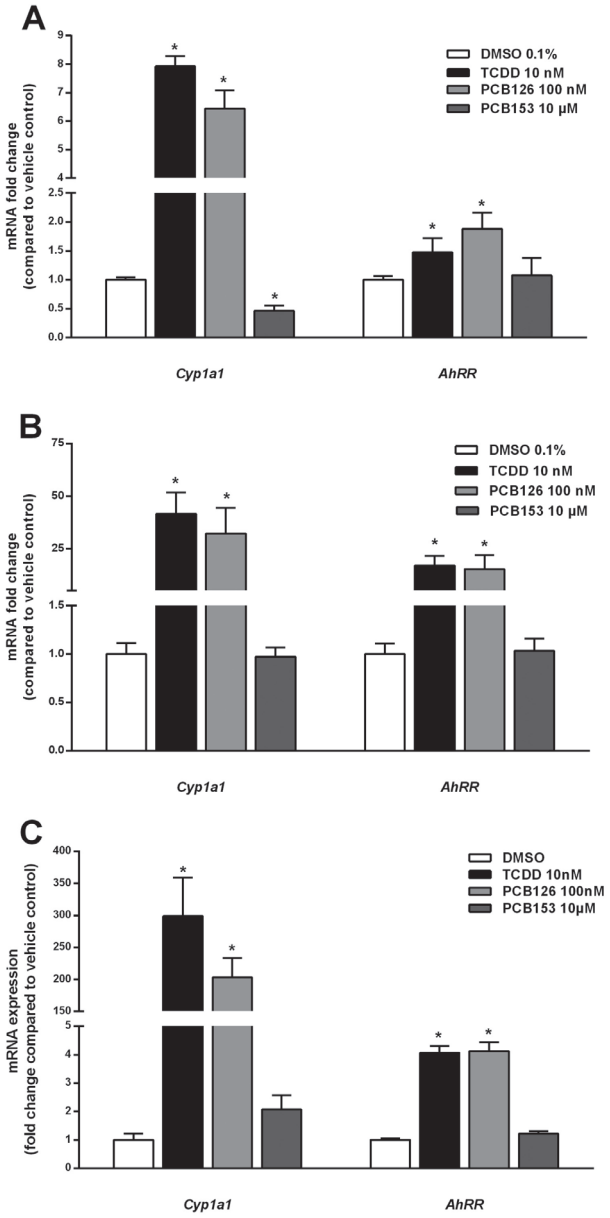

Fig.2 Gene expression of *Cyp1a1* and *AhRR* in rat primary culture of hypothalamus (A), pituitary (B) ovaria (C). Cells were treated with TCDD (10 nM), PCB126 (100 nM) and PCB153 (10 µM) for 24 hours. Data are expressed as fold-change compared with expression in vehicle-control treated cells. Bars represent mean+SEM of three independent experiments that were performed in triplicate (N=3). * $P < 0.05$ was considered statistically significant.

by TCDD, PCB126 and 153, respectively. Electrophysiological and imaging data have demonstrated that Kiss-1 neurons act at the hypothalamus level and regulate GnRH neuronal function [57; 58; 59]. It is also well-known that GnRH neurons have their intrinsic properties to release GnRH in a pulsatile manner [60]. Tamar et al. (2006) described daily fluctuations in *Gnrh1* and *Gnrhr* mRNA expression in the hypothalamus throughout the estrus cycle in normal female and also in ovariectomized (OVX) rats. TCDD was shown to influence daily fluctuations of *Gnrh1* via disturbance of the hypothalamic GABA-ergic neurons [29]. Thus indirect effects of dioxin and dioxin-like compounds on GnRH neurons cannot be excluded. In contrast, our recent study showed that exposure to TCDD (10 nM) significantly affected mRNA expression of *Gnrh1* in a time- and AhR-dependent manner in rat immortalized hypothalamic GnV-3 cells [31]. *Gnrh1* expression should be determined at multiple time-points in order to clarify whether dioxins are capable of disturbing the daily GnRH pattern in primary hypothalamic cultures.

Some studies have highlighted the sensitivity of Kiss-1 neurons as a target of endocrine modulation. In rat brain, the Kiss-1 neurons are organized in two different populations that are neuroanatomically separated within the hypothalamus; one is located in the arcuate nucleus (ARC) and another in anteroventral periventricular nucleus (APVN) [61; 62]. The latter population of Kiss-1 neurons has been demonstrated to be activated by estradiol and send axonal projections to GnRH neurons in the preoptic area (POA) suggesting that Kiss-1 neurons are potentially involved in conveying the estrogen (E2)-positive feedback [63;64]. The Kiss-1 population of neurons located in the ARC has been shown to give opposite responses to E2, as *Kiss-1* expression was inhibited by E2 [65; 66]. In our study, whole dissected rat hypothalami were cultured and used for further gene expression analysis. This means that no distinction was made between ARC and APVN neurons, which could have affected *Gnrh1* gene expression responses in our studies. Some other reports have shown that PCBs could interfere with the organization of the hypothalamus, particularly the AVPV [67]. Perinatal exposure to a mixture of PCBs i.e. Arochlor 1221, resulted in significant decrease in ERbeta-positive cell numbers in the AVPV and changes in the organization of the AVPV, causing masculinization of the female hypothalamic neurons [67]. Hence, any EDC with the capacity to interact with ER, or its expression, presumably has the greatest potential to disrupt the function of the kisspeptin system. Our research demonstrates that both dioxin and non-dioxin compounds may have an impact of Kiss-1 mRNA expression. Further time-point studies targeting specific Kiss-1 regions could markedly define dioxin impact on the regulation of Kiss-1/GnRH neurons.

2.2 Effects of dioxins at pituitary level

Proper folliculogenesis is maintained by the release of crucial pituitary gonadotropins FSH and LH, which synthesis is mainly driven by the specific patterns of pulsatile bursts of hypothalamic GnRH. FSH and LH mediate their action on ovary level through binding to specific cognate cell-surface receptors in granulosa and theca cells, respectively. To determine the effect of tested congeners on the alteration of gonadotropins FSH and LH, primary pituitary cultures were exposed to TCDD (10

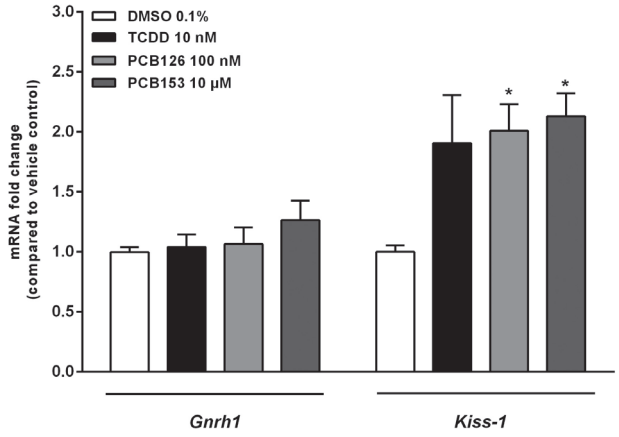

Fig.3 The expression of *Gnrh1* and *Kiss-1* in the primary rat hypothalamus cultures treated with TCDD (10 nM), PCB126 (100 nM) and PCB153 (10 µM) for 24 hours. The experiment was performed in triplicates and repeated three times (N=3). Data are expressed as fold-change compared with expression in vehicle-control treated cells. Bars represent mean+SEM of three independent experiments that were performed in triplicate (N=3). * $P < 0.05$ was considered statistically significant.

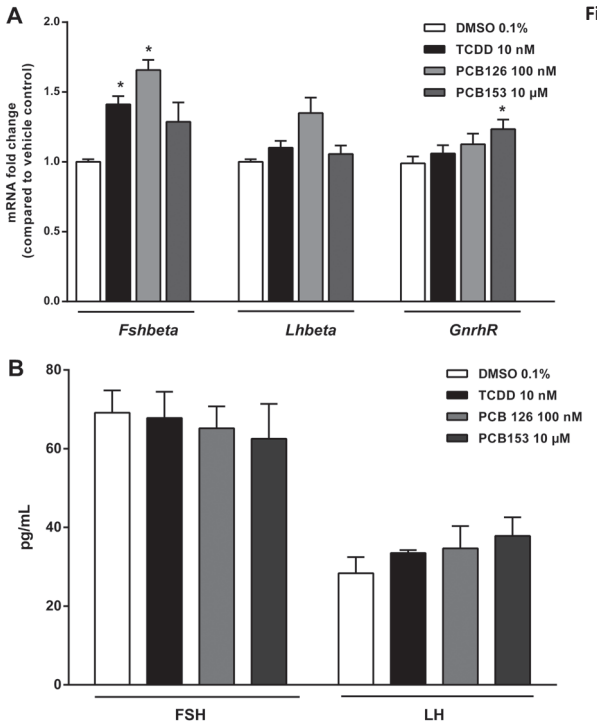

Fig.4 The expression of *Fshbeta, Lhbeta* and *GnrhR* mRNAs in the whole primary rat pituitary culture. The cells were treated with TCDD (10 nM), PCB126 (100 nM) and PCB153 (10 µM). The experiment was performed in triplicates and repeated three times (N=3). Data are expressed as fold-change compared with expression in vehicle-control treated cells. Bars represent mean+SEM of three independent experiments that were performed in triplicate (N=3). * $P < 0.05$ was considered statistically significant.

nM), PCB126 (100 nM) or the non-dioxin PCB153 (10 μM) for 24 hours. Both TCDD and PCB126, but not PCB153, significantly up-regulated the mRNA levels of *Fshbeta* rising its levels up to 1.4 and 1.7-fold, respectively (Fig.4A). Yet, the protein level of FSH remained unchanged after a 24-hour exposure (Fig.4B). The localization of AhR in the pituitary and *Fshbeta* mRNA increase by TCDD and PCB126 suggests that the AhR may play a role in this process. The levels of *Lhbeta* mRNA were not statistically significantly affected by the tested compounds, nor were the protein LH levels in the medium changed (Fig. 4A and B). Petroff et al. (2003) has demonstrated an increase of FSH and LH serum after exposure to TCDD in immature female rat model [68]. Similarly, others have shown a TCDD-induced acute release of FSH and LH in the immature gonadotropin-primed rat model [24; 69]. Also PCB126 was shown to increase the pituitary content of LH and FSH in male rats but interestingly a significant reduction of the plasma gonadotropins levels was detected. According to authors, this could be attributed to the alteration of the secretory mechanisms by PCB126 [70]. In the same study, PCB153 significantly decreased LH in plasma, but its content as well as levels of FSH in blood and pituitary remained unchanged [70].
It is well-known that fetal, neonatal, and immature animals are more sensitive to reproductive toxicants [71]. Mounting this evidence, the use of tissues derived from adult rats could result in less severe effects caused by dioxins. In rats, the levels of FSH and LH begin to increase and the surge of gonadotropins occurs during the late afternoon of proestrus. Presumably, the estrous status of the rat might have affected the effects on dioxins on gonadotropin regulation in our study. Overall, our observations support the notion that FSH is one of the targets of dioxin-like compounds in females. More functional analysis of the role of AhR in FSH regulation and potential dioxin effects with respect to the dynamic nature of these hormone secretion need to be performed to elucidate this further.

2.3 Effects of dioxins at ovary level

Several *in vivo* and *in vitro* studies have indicated the endocrine disrupting effects of dioxins at ovarian level, which are at least in part caused by direct action on this organ [33; 35; 72]. We used a primary rat ovarian granulosa-theca cell model to investigate the action of TCDD, PCB126 and 153 on ovarian theca and granulosa cell function. The transmembrane protein FSH receptor (FSHr) is present on granulosa cells while LH receptor (*Lhr*) expression is limited to the theca cells in preantral follicles. As oocyte development proceeds, granulosa cells of preovulatory follicles also express Lhrs [73]. Using qRT-PCR we demonstrated the expression of *Lhr* and *Fshr* in the granulosa-theca culture model (data not shown). As shown in Fig.5A, TCDD (10 nM) and PCB126 (100 nM) had a marked impact on *Fshr* by decreasing its mRNA levels to 32% and 23% of vehicle control-treated cell cultures, respectively. FSH (200 ng/mL) slightly up-regulated *Fshr* up to 1.3 fold (Fig.5A). Interestingly, LH (200 ng/mL) significantly decreased both *Fshr* and *Lhr* mRNA levels by 20% and 35%, respectively (Fig.5A;B). To further characterize the mechanisms underlying the interaction between gonadotropins and dioxins, we co-exposed cells with FSH (200 ng/mL) and tested congeners for 24 hours. The co-exposure to TCDD and PCB126 with FSH abolished the inhibitory effects of TCDD and PCB126 on *Fshr* mRNA.

PCB153 (10 µM) did not have an effect on *Fshr* mRNA. Also, all tested congeners did not exert modulatory effects on *Lhr* mRNA (Fig.5B). Our study demonstrated, that TCDD and PCB126 but not non-dioxin like PCB153 exert their modulatory effects on FSH-mediated pathway through the alteration of *Fshr* mRNA. Several studies have indicated a crosstalk between AhR and FSH pathways in the transcription of the Fshr gene expression as consequence of the presence of XRE in the promoter regions of the *Fshr* and an E-box binding site in the mouse ovary [74; 75; 76]. Also, the recent study of Teino *et al.* (2014) has shown that the expression of *Ahr* in mouse granulosa cells is modulated by gonadotropins during follicle development. More functional analysis of AhR-targeting e.g. using siRNA, need to be performed to explain dioxin-mediated downregulation of *Fshr* [77].

FSH plays an important role in the promotion of the expression of steroidogenic gene i.e. Cyp19a, involved in E2 synthesis in granulosa cells [78]. According to the two-cell/two-gonadotropin theory, ovarian steroidogenesis is being governed by pituitary gonadotropins whereby LH stimulates theca cells to produce androgens which are subsequently aromatized by FSH-stimulated granulosa cells. Surprisingly, the mRNA levels of *Cyp17* were not affected by LH (200 ng/mL), nonetheless LH induced *Cyp191a* up to 3 fold when compared to vehicle-treated control cells (Fig.6A,B). FSH significantly elevated *Cyp17* and *Cyp191a* mRNA levels up to 8 and 2.5 fold, respectively (Fig.6A,B). Our results are in line with the study of Hoang et al. (2013), who showed FSH (10 ng/mL) induced a significant increase of theca cell *Cyp17* and granulosa cell *Cyp191a* mRNAs in immature female rats after 24 and 48 hours of exposure [79]. However, in our study, the 24 and 72 hour exposure to TCDD did not affected the E2 protein levels (Fig.6C). We did observe an unexpected, but consistent, increase in E2 levels up to 15 and 23% after 24 and 72 hour-treatment with PCB126 (100 nM) compared to vehicle control. Previous *in vivo* studies have showed the disrupting effects of dioxins on E2 signaling through the alteration of ER mRNAs and declining E2 protein levels [24]. The reversible decrease of E2 secretion by TCDD was demonstrated in the human lutenized granulosa cells (LhGC) for 8, 12 and 24 hours, after that the levels of E2 increased after 36 and 48 hours [72]. Contrary, others failed to demonstrate changes in E2 serum concentrations after treatment with TCDD [68]. The latter results are in line with our study. There are a number of reports implicating TCDD and PCBs in the non-genomic type of actions of ligand-activated AhR. Indeed the increase of E2 levels after exposure to PCB126 after 24 and 72 hours may suggest the involvement of non-genomic pathways in the modulation of steroid function by this dioxin like compound. It has been also documented that AhR activation can result in the inhibition or promotion of steroid hormone signaling in reproductive tissues and this may, in part, can explain the contradictory results of estrogenic or anti-estrogenic effects mediated by AhR ligands. Future research on dioxins should therefore be more directed towards the mechanism of non-genomic action of dioxin-like compounds.

In the present study the concentrations of tested congeners clearly exceeded the levels observed in the human body. Nonetheless, because of daily exposure of these lipophilic compounds and their easy bio-accumulative nature in fat-rich organs like ovaries, it can not be excluded that the excessive levels of dioxins may

occur. Since humans and animals are exposed to a mixture of dioxins and other environmental toxicants, it could be important in the future to study the toxicity of these environmentally relevant mixtures on HPO-axis.

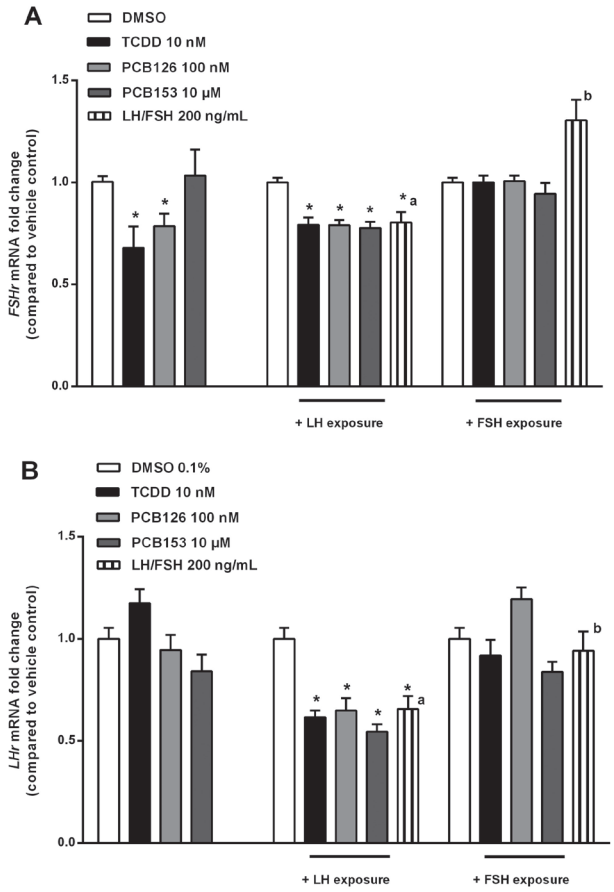

Fig. 5 The expression of *Fshr* (A) and *Lhr* (B) mRNAs in the whole primary rat ovaria culture. The cells were treated with TCDD (10 nM), PCB126 (100 nM) and PCB153 (10 µM), LH (200 ng/mL) (a) and FSH (200 ng/mL) (b) for 24 hours. The experiment was performed in triplicates and repeated three times (N=3). Data are expressed as fold-change compared with expression in vehicle-control treated cells. Bars represent mean+SEM of three independent experiments that were performed in triplicate (N=3). * significant from DMO-vehicle control and # significant from positive controls (LF or FSH, respectively). $P < 0.05$ was considered statistically significant.

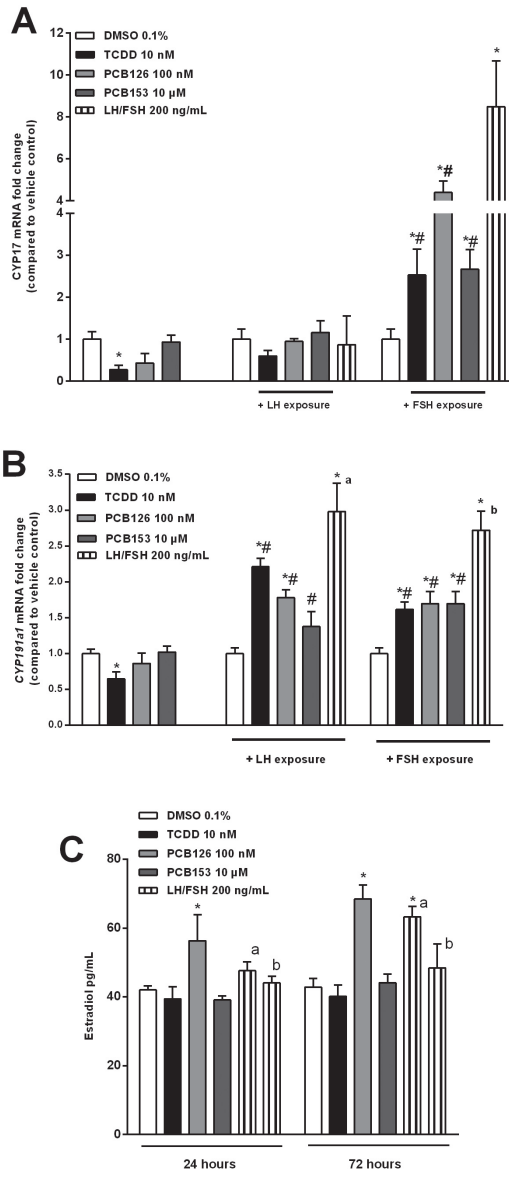

Fig. 6 The expression of *Cyp17* (A) and *Cyp19a1* (B) mRNAs in the whole primary rat ovaria culture treated with TCDD (10 nM), PCB126 (100 nM) and PCB153 (10 µM), LH (200 ng/mL) (a) and FSH (200 ng/mL) (b) for 24 hours. Data are expressed as fold-change compared with expression in vehicle-control treated cells. Bars represent mean+SEM of three independent experiments that were performed in triplicate (N=3). The levels of E2 measured in primary rat ovaria cultures after 24 and 72 hours after the exposure to TCDD (10 nM), PCB126 (100 nM) and PCB153 (10 µM), LH (200 ng/mL) (a) and FSH (200 ng/mL) (b) (C). The experiment was performed in triplicates and repeated two times (N=2). * significant from DMO-vehicle control and # significant from positive controls (LF or FSH, respectively). $P < 0.05$ was considered statistically significant.

Conclusion

Our study demonstrates that dioxin-like compounds such TCDD and PCB126 can directly target all compartments of the HPO-axis *in vitro*. The results presented in this study support *in vivo* animal data on dioxin-like compounds in reproductive system and provide additional information with respect to mechanistic action of these toxic compounds on HPO system.
Non-dioxin like PCB153 did not exert such pronounced effects on reproductive axis. The action of TCDD and PCB126 on steroid hormone gene expression are likely through an activation of AhR pathway, as demonstrated by induction of the *Cyp1a1* transcripts in all culture systems. The largest effects of the dioxin-like compounds were observed in the ovarian cultures. The AhR-mediated actions of dioxins can be linked to modulation of the Fsh signaling at pituitary and ovary level. Less pronounced effects were observed at hypothalamus. Here, we show that Kiss-1 neurons can be dioxin targets and might serve as potential bio-markers of dioxin exposure in the brain. The use of primary tissue cultures derived directly from healthy animal tissue could make an advantage over the commercially available cancer-derived cell lines and may contribute to better understanding of dioxin and non-dioxin compounds mode of action. This study presents the first step in characterization of dioxin-like effects upon HPO-axis *in vitro*. Next challenge is to apply co-culture primary tissue models to better introduce crucial hormonal feedback mechanisms tightly regulating reproductive axis *in vivo*.

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Supplementary Table 1. Primers used for qRT-PCR. The asterisk (*) indicates a gene used as reference normalizer gene.

Gene name (accession nr.)	Forward primer	Reverse primer	PCR product [bp]
actin, beta (Actb)* NM_031144.2	AGCGTGGCTACAGCTTCACC	AAGTCTAGGGCAACATAGCACAGC	85
aryl-hydrocarbon receptor repressor (Ahrr) NM_001024285.1	CCCCAAGGGGACTTCAGGGGAC	TGCTCCAGTCCAGGTGCCTCA	146
cytochrome P450, family 1, subfamily a, polypeptide 1 (Cyp1a1) NM_012540.2	TCACCATCCCCCACAGCACCA	TCAGGCCGGAACTCGTTTGGA	139
Gonadotropin-releasing hormone 1 (Gnrh1) NM_012767	GGCAAGGAGGAGGATCAAA	CCAGTGCATTACATCTTCTTCTG	142
gonadotropin releasing hormone receptor (Gnrhr) NM_031038.3	GGCAAGGAGGAGGATCAAA	CCAGTGCATTACATCTTCTTCTG	142
Kiss-1 metastasis-suppressor (Kiss1) NM_181692.1	ACCCACAGGCCAACAGTCCG	ATGGCGATGTTCGGCGAGCG	116
luteinizing hormone beta (Lhb) NM_012858.2	ACCTTCACCACCAGCATCTGT	AGCTCACGGTAGGTGCACACT	104
Follicle stimulating hormone, beta polypeptide (Fshb) NM_001007597.1	CTGGTGCTTGAGAGCAGTCTGC	CACCAGATCCCTGGTGTAGCAGTA	139
Luteinizing hormone/choriogonadotropin receptor (Lhcgr) NM_012978.1	AAACGCCGGGCGGAGCTTTA	TCAGGGTAGCCTGGGACGGC	103
follicle stimulating hormone receptor (Fshr) NM_199237.1	TGCTGGCATTCTTGGGCACGG	AATGGCGTTCCGGGGGAGGT	125
cytochrome P450, family 19, subfamily a, polypeptide 1 (Cyp19a1) NM_017085.2	GCCTGTGGAGAACGGTCCGC	ACCACGTCCACGTAGCCCGA	148
cytochrome P450, family 17, subfamily a, polypeptide 1 (Cyp17a1) NM_012753.1	TCCTGGTGCACAATCCTGAGGTGA	CCACCGGCCTGATACGCAGC	150

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CHAPTER 3

In vitro neuroendocrine effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the AhR-expressing hypothalamic rat GnV-3 cell line.

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Abstract

The aryl hydrocarbon receptor (AhR) is involved in a wide variety of biological and toxicological responses, including neuroendocrine signaling. Due to the complexity of neuroendocrine pathways in e.g. the hypothalamus and pituitary, there are limited *in vitro* models available. This in spite of the strong demand for such systems to study and predict neuroendocrine effects of chemicals. In this study, the applicability of the AhR-expressing rat hypothalamic GnV-3 cell line was investigated as a novel model for screening of neuroendocrine effects of AhR ligands using 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as reference compound. The qRT-PCR analyses demonstrated the presence of several sets of neurotransmitter receptors in the GnV-3 cells. TCDD (10 nM) altered neurotransmitter signaling by up-regulation of glutamate ($Grik_2$), gamma-amino butyric acid ($Gabra_2$) and serotonin (Ht_{2c}) receptor mRNA levels. However, no significant changes in basal and serotonin-evoked intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) or serotonin release were observed. On the other hand, TCDD de-regulated Period circadian protein homolog 1 (*Per1*) and Gonadotropin releasing hormone (*Gnrh*) mRNA levels within a 24-hour time period. Both *Per1* and *Gnrh* genes displayed a similar mRNA expression pattern in the GnV-3 cells. Moreover, the involvement of *AhR* in TCDD-induced alteration of Neuropeptide Y (*Npy*) gene expression was found and confirmed by using siRNA targeted against *Ahr* in GnV-3 cells. Overall, the combined results demonstrate that these GnV-3 cells may be a suitable model to predict some mechanisms of action and effects of AhR ligands in the hypothalamus.

1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to the basic helix–loop–helix Per–Arnt–Sim (PAS) superfamily, which in response to exogenous and/or endogenous chemicals modulates a diverse battery of genes. The AhR plays a multi-functional role in various cellular processes, including cell differentiation during organ development, regulation and maintenance of cell cycle signaling, government of circadian rhythms and activation of stress response pathways (Gu et al., 2000; Massari and Murre, 2000, Kewley et al., 2004; Denison et al., 2011). A dormant, soluble form of AhR is located in the cytoplasm. Upon ligation, a cascade is initiated that involves dissociation of heat shock proteins, translocation of the AhR receptor–ligand to the nucleus and association of the ligand-AhR complex with its dimerization partner Aryl Hydrocarbon Receptor Nuclear translocator (ARNT) to dioxin responsive elements (DREs) on the DNA. This cascade finally results in increased transcription of a myriad of genes, including cytochrome P-450 enzymes e.g. Cyp1A1, 1A2, and 1B1 and several phase II enzymes genes (Poland et al, 1974, Kohn et al., 1993, Nebert et al.,1993).

The induction of cytochrome P-450 enzymes after exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a potent AhR activator, has been shown to occur in many tissues including various regions of the rat brain (Kainu et al, 1995; Unkila et al., 1993a; Huang et al., 2000). Upon oral exposure of rats to TCDD, the highest concentration in the brain has been found in the hypothalamus (Kainu et al., 1995; Korkalainen et al, 2005). In the olfactory bulb, hippocampus, cerebral and cerebellar cortices, AhR is co-expressed together with ARNT (Kainu et al., 1995; Huang et al, 2000). Also, high levels of mRNA encoding AhR were identified in specific hypothalamic regions known to be involved in the regulation of food intake and circadian hormone fluctuations in the adult rat brain. (Petersen et al., 2000). The hypothalamus is located at the base of the brain and consists of diverse neurons that control central neuroendocrine functions and serves as an essential link between the CNS and the rest of the body. Moreover, this part of the brain is believed to be the main site for serotonergic action on feeding behavior and body weight regulation. Weight loss and progressing anorexia are hallmarks of both acute and chronic TCDD intoxication in rodents (Bestervelt et al, 1991; Tuomisto et al, 1999) and exposure to this dioxin has been linked to changes in mRNA levels of hypothalamic orexigenic and anorexigenic neuropeptides (Fetissov et al., 2004). Additionally, exposure to TCDD has been reported to affect the turnover of several neurotransmitters and their receptors involved in GABA-ergic, glutaminergic and serotonergic signaling in rodents (Hays et al., 2002; Kakeyama et al., 2003; Unkila et al., 1993a,1995b). The latter is known to affect satiety and has been implicated in the reduction of food intake. A single exposure to TCDD was shown to disrupt circadian rhythm- controlled genes such as *Per1* and Brain and muscle Arnt-like protein 1 (*Bmal1*) in the central clock located in the suprachiasmatic nucleus (SCN) of the rat hypothalamus (Xu et al., 2010).

Whereas the steps between TCDD binding and transcriptional activation of many AhR target genes are well understood, little is known about the downstream molecular

events that lead to the observed TCDD effects at the hypothalamus level. Moreover, complex cellular interactions within the hypothalamus limit the possibility to study adverse effects of exogenous compounds at the cellular and molecular level *in vivo* and *ex vivo*. For mechanistic and toxicological purposes, a good *in vitro* model for the hypothalamus is therefore of great value. While most *in vitro* studies that investigate the hypothalamus use the murine GT1-7 cell line, this cell line does not express the AhR (Petroff et al., 2003). Recently, a novel rat hypothalamic cell line GnV-3 was described as a useful novel tool for the study of hypothalamic GnRH neurons at cellular and molecular levels (Salvi et al., 2006). GnV-3 cells were generated by a modified Tet-On system to condition the expression of the immortalizing agent *v-myc* to the presence of doxycycline in the culture medium (Salvi et al., 2006). This allows proliferation of GnV-3 cells in the presence of doxycycline, whereas in the absence of doxycycline, the cells exhibit many features of mature, non-dividing, differentiated GnRH neurons (Salvi et al., 2006; Mansuy and Bujard, 2000). In contrast, most available GnRH-releasing hypothalamic cell lines, such as GT1-7, were generated from developing murine *GnRH* neurons using the SV-40 large T antigen transformation system targeted to the Gnrh promoter region, and therefore may not entirely resemble the differentiated adult neurons due to loss of the post-mitotic stages (Efrat et al., 1988).

The present study was designed to determine the presence of a functional AhR signaling pathway in the novel hypothalamic GnV-3 cell line. In addition, neuroendocrinological effects of AhR activation were determined on a cellular and molecular level by using TCDD as known potent AhR agonist. To our knowledge, this is the first study demonstrating new neuroendocrinological and classical AhR-mediated cellular and molecular effects in a hypothalamic cell line.

2. Materials and Methods

2.1 Cell culture

Rat hypothalamic GnV-3 cells (established in the Department of Medicine, University Hospital, Lausanne, Switzerland), were cultured as previously described (Salvi et al., 2006, Mansuy et al., 2011). Briefly, cells were grown on 5 µg/ml poly-D-lysine-coated flasks (Sigma-Aldrich, Fluka Chemie) in a proliferation medium, consisting of neurobasal A containing phenol-red (Invitrogen-Gibco) with 2% B27 supplement (50X) (Invitrogen-Gibco), 1% heat-inactivated charcoal-stripped FBS, 200 mM GlutaMax (Invitrogen-Gibco), 25 µM L-glutamate (Sigma-Aldrich Fluka Chemie), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 ng/ml basic Fibroblast Growth Factor (bFGF) (Invitrogen) and 5 µg/ml doxycycline (Sigma-Aldrich, Fluka Chemie). For all experimental conditions, GnV-3 cells were seeded onto 5 µg/ml poly-D-lysine-coated flasks and cultured for 3 days in differentiation phenol red-free Neurobasal medium supplemented without doxycycline to stop cell proliferation and to develop the features of mature GnRH neurons (Mansuy et al., 2011). GnV-3 cells were used between passage number 19-24. Half of the medium was changed during each passage cycle. Cells were harvested every week at 90-95% confluence. Liver murine

H1L7.5c3 cells were obtained from American Type Culture Collection (ATCC) and cultured in MEM alpha (Invitrogen-Gibco) supplemented with 10% FBS and were subcultured twice a week. Murine hepatic Hepa1C17 cells were cultured according to the protocol provided by ATCC. The hypothalamic murine GT1-7 cells (kindly provided by Prof. Pamela Mellon, University of California, San Diego) were grown in DMEM (Invitrogen-Gibco) supplemented with 10% FBS and were subcultured once a week. All cells were cultured at 37°C in a humidified atmosphere at 5% CO_2.

2.2 Chemicals and reagents

2,3,7,8-tetrachlorodibenzodioxin (TCDD) was purchased from Wellington Laboratories Inc. (Guelph, Ontario, Canada) and dissolved in dimethyl sulfoxide (DMSO). Forskolin was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and dissolved in DMSO. NaCl, KCl, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Merck (Whitehouse Station, NJ, USA); MgCl2, CaCl2, glucose, sucrose, and NaOH were obtained from BDH Laboratory Supplies (Poole, U.K.); and Fura-2 AM and H_2-DCFDA were obtained from Molecular Probes (Invitrogen, Breda, The Netherlands). Serotonin (5-HT) (Sigma-Aldrich, Zwijndrecht, The Netherlands) was dissolved in external saline buffer containing (in mM): 125 NaCl, 5.5 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, 24 glucose, and 36.5 sucrose at pH 7.3, adjusted with NaOH) in deionized water (Milli-Q; resistivity> 10 MΩ·cm).

2.3 Cell viability (Alamar Blue (AB), Neutral Red (NR)) and Reactive Oxygen Species (ROS) assay

GnV-3 cells (1.25 x 105) were seeded onto 5 µg/ml poly-D-lysine-coated 24-well plates in phenol red-free Neurobasal differentiation medium. Briefly, GnV-3 cells were exposed to different concentrations of TCDD (0.1-10 nM) for 24 hours. The AB (Invitrogen, Basel, Switzerland) and NR (Sigma-Aldrich (Zwijndrecht, The Netherlands) assays were performed subsequently on the same set of plates. After a 1.5-hour incubation at 37°C with AB solution (1:20), the redox reaction in which AB was reduced by the GnV-3 cells was measured by absorbance readings at 540 and 595 nm. Then, cells were washed once with differentiation medium and the NR (50 µg/mL) was added to each well for another 1.5 hour at 37°C. Following incubation with NR, 500 µl extraction solution (1% glacial aceticacid, 50% ethanol and 49% H_2O) was added to the wells. After 15 min of extraction, the quantity of dye incorporated into cells was measured at 530/645 nm (excitation/emission wavelength). The production of ROS was assessed using the fluorescent dye H2-DCFDA as described previously (Hendriks et al., 2012). Briefly, GnV-3 cells (1.5 x 10^5) seeded in 12-well plates were loaded with H2-DCFDA (1.5 µM) for 30 min at 37°C. Subsequently, cells were exposed to different concentrations of TCDD (0.1-10nM) for 24 hours. ROS production was measured spectrophotometrically as an increase in fluorescence at 485/530 nm (excitation/emission wavelength). All measurements were performed using Tecan infinite M200 (Tecan Group Ltd, Männedorf, Switzerland).

2.4 Protein extraction and Western Blot analysis

GnV-3 cells (6 x 10^6), murine hypothalamic GT1-7 cells (10 x 10^6), murine liver Hepa1C17 cells (2 x 10^6), murine liver HL1.5c3 cells (10 x 10^6) were washed once with ice-cold PBS before adding the ice-cold lysis buffer (NaCl (150mM), TRIS-HCl (20mM), glycerol (5%), EDTA (2mM), Triton-X (1%), aprotinin (50µg/mL), PMSF (1mM)). The total protein concentration of lysates was quantified using the Lowry assay (Lowry et al., 1951). Twenty and ten micrograms of cell protein and protein standards were fractionated by SDS-PAGE (10%), electrophoretically transferred onto polyvinylidene fluoride (pvdf) membrane and probed for 1 hour at room temperature (RT) with a primary polyclonal goat anti-mouse AhR antibody recommended for detection of mouse and rat AhR, dilution factor 1:250 (Santa Cruz, Biotechnology). After washing, the membranes were incubated for another 1 hour at room temperature (RT) with a secondary polyclonal donkey anti-goat AP-conjugated antibody, dilution factor 1:2000 (Santa Cruz, Biotechnology). Protein bands were visualized using the 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) substrate system (Sigma St. Louis, MO, USA). Precision Plus Protein™ All Blue Standards were used at the 10-250 kDa range as markers (Bio-Rad Laboratories, Inc.).

2.5 AhR Immunostaining

GnV-3 cells (5 x 10^5) were grown in differentiation medium on 50 µg/ml rat tail collagen (RTC) coated glass coverslips. Cells were allowed to adhere for 48 hours. GnV-3 cells were exposed to TCDD (10 nM) for 1, 2 and 6 hours. After exposure to TCDD, cells were rinsed with PBS and fixed in 4%-buffered paraformaldehyde (pH 7.4) at RT for 30 min. Fluorescent microscopy studies were performed using an Olympus BX60 fitted to a Leica DFC425C camera with the following antibodies: primary AhR antibody, dilution factor 1:50 (Santa Cruz, Biotechnology) for 90 min at RT, and then secondary donkey anti-goat HRP-conjugated antibody (Santa Cruz, Biotechnology), dilution factor 1:100 for 60 min at RT. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, the Netherlands) for 10 min.

2.6 Quantitative real time polymerase chain reaction (RT-PCR)

GnV-3 cells (2.5 x 10^5 cells) were seeded onto 5 µg/ml poly-D-lysine-coated 12-well plates in differentiation phenol red-free Neurobasal medium. Total RNA was harvested from the GnV-3 cells by phenol–chloroform extraction using RNA Instapure (Eurogenetic, Liege, Belgium). Purity and concentration of the RNA samples were determined spectrophotometrically at an absorbance wavelength of 260/280nm and 230/260 nm. Complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis Kit, according to the manufacturer's instructions (Biorad, Veenendaal, the Netherlands). Obtained cDNA was diluted 10 times and was stored at -20 °C until further analysis. The primers, if not indicated, were designed using Primer-BLAST of National Center for Biotechnology Information (NCBI) and then checked with BLAST (nucleotide nonredundant database) to confirm specificity. The primers for *Per1* and *Gnrh* were described elsewhere (Mansuy et al., 2009; Schirman-Hildesheim et al., 2005). The primer pairs used in the qRT-PCR are listed in the supplementary data Table S1.

2.7 siRNA against rat Ahr

GnV-3 cells (1,5 x 10^5) were seeded onto 5 µg/ml poly-D-lysine-coated 12-well plates and allowed to attach in differentiation medium and reach 80% confluency. Transient transfection was performed according to the manufacturer's instructions in a total volume of 600 µl containing 1µL/well Lipofectamine 2000/ RNAiMAX (Invitrogen, the Netherlands), 5nM siRNA directed against rat Ahr mRNA (sense siRNA: CGUUAGAUGUUCCUCUGUGtt, antisense siRNA: CACAGAGGAACAUCUAACGtt, as previously described (Weiss et al., 2008). 5 nM of Stealth RNAi™ Hi GC (Invitrogen, the Netherlands) was used as a negative control. 48 hours after transfection, GnV-3 cells were exposed to TCDD (10 nM) for 4, 6 and 24 hours in the Lipofectamine-containing medium.

2.8. Serotonin (5-HT) detection in GnV-3 cells

GnV-3 cells (5 x 10^4) were seeded onto 5 µg/ml poly-D-lysine coated 96-well plates and allowed to attach for 48 hours in differentiation medium. Cells were pre-exposed to TCDD (10 nM) and DMSO (0.1% v/v) for 2 hours. After that, media were removed and cells were exposed again to TCDD (10 nM) and DMSO (0.1%) for 1hour. The media were then collected and immediately stored at -80°C until use. Serotonin high sensitive ELISA kit was purchased from IBL International (GmbH, Hamburg, Germany). The experiment was performed in triplicate. Measurement of 5-HT in media was conducted according to the manufacturer's instruction using Tecan infinite M200 (Tecan Group Ltd, Männedorf, Switzerland).

2.9 Fluorescent Ca^{2+} imaging

GnV-3 cells (3 x 10^5) were plated onto 50 µg/ml RTC-coated glass bottom dishes (MatTek, Ashland MA, USA) three days prior to experiments. Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) were measured in cell loaded with the Ca^{2+}- sensitive fluorescent ratio dye Fura-2AM (7.5 µM; Invitrogen, Breda, The Netherlands) as described previously (Langeveld et al, 2012). During experiments, cells were continuously superfused with saline at a rate of ~0.6 ml/min using a ValveLink 8.2 perfusion system (AutoMate Scientific, Berkeley, CA). Fluorescence, evoked by 340 and 380 nm excitation wavelengths (F340 and F380, respectively), was collected every 3 s at 510 nm using an Image SensiCam digital camera (TILL Photonics GmBH). Experiments to determine acute effects of TCDD on $[Ca^{2+}]i$ consisted of a 10-min baseline recording to measure basal $[Ca^{2+}]i$, after which an increase in $[Ca^{2+}]i$ was evoked by changing the superfusion to saline containing 100 µM 5-HT for 15s. Following this first stimulation and a ~9-min recovery period, GnV-3 cells were exposed to 0.1% DMSO-containing saline (control) or 10 nM TCDD-containing saline for 15 min prior to a second stimulation with 100 µM 5-HT (see Figs 6A and B for example recordings). In a separate set of experiments, GnV-3 cells were pre-exposed to 10 nM TCDD for 0-24 hours to determine effect of prolonged TCDD exposure on basal and 100 µM 5-HT-evoked in $[Ca^{2+}]i$ (see Figs 6C and D for example recordings). All experiments were performed at RT.

2.10. Data analysis and statistics

Experiments were performed in three independent experiments that were performed in triplicate, unless stated otherwise. Statistically significance in difference of the mean were calculated using one- or two-way ANOVA followed by Dunnett's post-test. Data were analyzed using Prism 6.0 (Graph Pad Software, San Diego, CA, USA) and Statistical Package for the Social Sciences (SPSS) software. For each experiment, differences were considered statistically significant at *P* values <0.05.

Ca^{2+} imaging data were analyzed using custom-made MS Excel macros as described previously (Langeveld et al, 2012) and free cytosolic $[Ca^{2+}]i$ was expressed as (normalized) F340/F380 ratio (R). To determine acute effects of TCDD exposure on the 5-HT-evoked increase in $[Ca^{2+}]i$, the amplitude of the second serotonin-evoked increase in $[Ca^{2+}]i$ (after 15 min of TCDD exposure) was expressed as a percentage of the amplitude of the first 5-HT-evoked increase in $[Ca^{2+}]i$ per cell to obtain a 'treatment ratio' (TR) as described previously (Langeveld et al, 2012). As the SD for basal $[Ca^{2+}]i$ and the first 5-HT-evoked increase in $[Ca^{2+}]i$ amounted to ~8 and ~14%, respectively, effects < 15% were considered irrelevant; all relevant effects are statistically significant ($p < 0.05$).

3. Results and Discussion

3.1 AhR system in the GnV-3 cell line

Since the hypothalamus plays an important role in neuroendocrine signaling *in vivo*, the presence of an active AhR pathway would be an important feature of a hypothalamic cell line. Western blot analysis confirmed the presence of AhR protein in GnV-3 cells, but not in GT1-7 cells (see Supplementary data Fig.S1.). As a positive control for AhR protein expression, two murine hepatic cell lines Hepa1C17 and HL1.5c3 were included. In order to compare possible changes in AhR expression due to different culture conditions, AhR protein was determined with GnV-3 cells that were grown in doxycycline-containing medium (proliferation medium) and doxycycline-free differentiation medium (assay medium) for 3 days. Mansuy et al. (2011) showed that in GnV-3 cells cultured without doxycycline, highly specific neuronal cell differentiation genes and genes involved in GnRH neuronal activity and cell cycle are over-expressed. In our study, AhR protein expression in GnV-3 cells cultured in differentiation medium was comparable to expression in GnV-3 cells cultured with doxycycline (data not shown). Immunostaining for AhR indicated that in the same culture dish, not all GnV-3 cells expressed the AhR protein which was likely due to the differentiation of the neurons. In AhR-expressing GnV-3 cells, TCDD exposure resulted in nuclear translocation of the AhR protein (10 nM; Fig.1B, D and F). Nonetheless, the presence of AhR in the nuclei was still observed after 24-hour of TCDD exposure, although its abundance was less prominent as compared to its sub-cellular compartment (Fig.1F). This phenomenon may be explained by the fact that TCDD can induce a shortening of AhR half-life through induction of proteasomal degradation (Ma and Baldwin, 2000), which may differ in time between individual cells. Using qRT-PCR we confirmed the presence of the Ahr system in the GnV-3 cells,

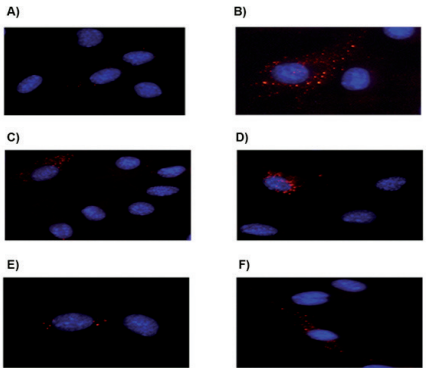

Fig. 1. AhR immunostaining of GnV-3 cells pre-treated with A) C) E) DMSO (0.1% v/v) or B) D) F) TCDD (10 nM) for 1, 2 and 24 hours, respectively. Cell nuclei were visualized by DAPI staining. All Images were taken with magnification 40X.

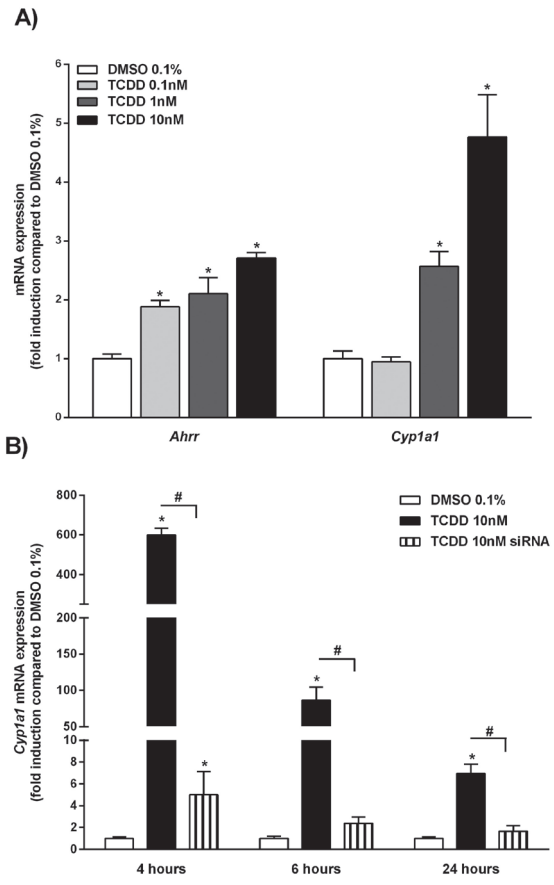

Fig. 2. A) Changes in mRNA levels of *Ahrr* and *Cyp1a1* in GnV-3 cells exposed to various concentrations of TCDD (0.1-10 nM) for 24 hours. Data are expressed as fold-change compared with expression in vehicle-control treated cells. Bars represent mean+SEM of three independent experiments that were performed in triplicate (N=3); B) Time-dependent changes in mRNA expression of *Cyp1a1* in Gnv-3 cells exposed to DMSO (0.1%, white bars) TCDD alone (10 nM, black bars) or TCDD (10 nM) with lipofectamine-transfection siRNA against *Ahr* (striped bars). Data are expressed as fold-change compared with expression in vehicle-control treated cells at the same time. Bars represent mean+SEM of four independent experiments that were performed in triplicate. *Significantly different from vehicle control-treated cells, P <0.05. #significantly different from TCDD (10 nM)-exposed cells.

including *Ahr*, but also the Aryl hydrocarbon receptor repressor (*Ahrr*), *Arnt1/2* (data not shown).

The mRNA analysis showed that activation of AhR by TCDD resulted in a concentration-dependent increase in expression of *Cyp1a1* and *Ahrr*, two sensitive hallmarks of AhR activation (Fig.2B). The induction of *Cyp1a1* was time-dependently affected by TCDD (10 nM; Fig.2B). Cyp1a1 expression was up-regulated 600-fold by TCDD after 4 hours, and then slowly decreased to about 7-fold higher after 24 hours compared to expression in vehicle control-treated cells. Knocking down *Ahr* with siRNA resulted in a 56% reduction of the AhR protein expression compared to negative (mock) control (data not shown). Concomitantly, induction of *Cyp1a1* mRNA expression by TCDD was negated. *Cyp1a1* expression was significantly reduced by 94, 97 and 76% compared to non-siRNA treated cells exposed to TCDD (10 nM), after 4, 6 and 24 hours, respectively (Fig.2B). Only after 4 hours, a statistically significant 5-fold induction of *Cyp1a1* mRNA by TCDD could still be observed in *Ahr* knockdown cells. No changes in *Cyp1a1* gene expression were observed in both control siRNA *Ahr*-mock cells and non-transfected negative control cells (data not shown). Taken together, our data clearly demonstrate the presence of an active and inducible *AhR* pathway in this GnV-3 cell line.

3.2. Cytotoxicity and oxidative stress

In rats and mice, TCDD exposure has been shown to cause an increase of oxidative stress in the brain. This is accompanied by enhanced production of superoxide anion and the induction of lipid peroxidation and DNA damage that finally leads to tissue damage (Bagchi et al., 2002; Hassoun et al., 1998). Here, we assessed cytotoxicity and production of reactive oxygen species (ROS) in GnV-3 cells upon TCDD exposure (1 nM-10 nM). No cytotoxicity was recorded for all tested concentrations of TCDD using Alamar Blue (AB) as well as Neutral Red (NR) cytotoxicity assays. Similarly, a 24-hour TCDD exposure (10 nM) did not result in significant changes in ROS production in GnV-3 cells (see Supplementary data Fig.S.2). Others have described that in neuroblastoma N2A cells TCDD only induced cytotoxicity at concentrations greater than 10 nM (Sul et al., 2009). In addition, the use of the antioxidant supplement B-27 in our culture medium possibly protected the GnV-3 cells against TCDD-induced ROS formation. These results show that in our subsequent experiments with GnV-3 cells, the effects of TCDD are not confounded by oxidative stress or cytoxicity.

3.3. Gene expression of neuroendocrine signals in the hypothalamic GNV-3 cell line

GnRH neurons possess an intrinsic GnRH pulse generator and are able to episodic GnRH pulse release in the absence of extrinsic circadian signals. This unique GnRH pulsatility can also be modulated by exogenous neuro-humoral stimuli (Halász et al., 1989; Chappell et al., 2010). Mounting evidence suggests that the endogenous circadian clock, with Per1 as a key player, plays a role in the regulation of GnRH pulse release (Chappell et al., 2010) and that circadian mechanisms are involved in timing gonadotropin activation to a specific phase of the 24 hour-day (De la Iglesia and Schwartz, 2012). Moreover, this circadian molecular clock was shown to play an

important role in the control of energy homeostasis *in vivo* (Kohsaka et al., 2007; Turek et al., 2005). Thus, any perturbation of metabolic homeostasis including hypothalamic feeding-related neuropeptides may contribute to the alteration of the clock gene function (Huang et al., 2011).

3.3.1. Per1/Gnrh gene expression

Salvi et al. (2006) showed that GnV-3 cells display an intrinsic pattern of GnRH release. In the present study, we also show the time-dependent fluctuations of *Gnrh* mRNA levels (Fig.3A). Interestingly, the *Per1* mRNA pattern oscillated in a comparable fashion with *Gnrh* (Fig.3A). *Per1* is known as a crucial regulator of the circadian rhythm that governs endogenous rhythmicity of all the cells in the body. As described by Olcese *et al.* (2003), GnRH neurons express *Per1* protein . The diurnal variation in expression of the clock genes including *Per1* were previously studied in rat hypothalamus and were shown to be involved in the regulation of daily rhythms of adrenocorticotrophins (ACTH), gonadotrophins and metabolic hormones (Girotti et al., 2009; Kalsbeek et al. 2006). Our study provides for the first time the evidence of synchronized pattern of *Per1* and *Gnrh* gene expression in an *in vitro* cell model. Moreover, this time-dependent expression pattern in GnV-3 cells can be altered by TCDD-mediated AhR activation (Fig.3B). This is in line with *in vivo* studies showing an alteration of circadian rhythm upon TCDD treatment (Tischkau et al., 2011; Garrett and Gasiewicz, 2006). In GnV-3 cells, exposure to TCDD (10 nM) significantly affected mRNA expression of *Gnrh* and *Per1* genes in a time-dependent manner. The observed effects of TCDD exposure on *Per1* gene expression were transient. The largest effect was seen after a 6-hour exposure to TCDD with a 1.4-fold induction of *Per1* expression compared with vehicle control-treated cells (Fig.3B). It could be argued, that as a member of the PAS domain family of transcription factors, AhR and circadian clock proteins share structural similarities. Earlier studies have shown that the *AhR* has no effects on the *in vivo* biological clock in the absence of exogenous agonist, whereas upon AhR activation circadian rhythmicity and clock gene expression were altered (Xu et al., 2010). Our observation of *Per1* modulation via *AhR* activation with TCDD remains elusive and needs further investigation from a mechanistic point of view. The largest effect of TCDD exposure on Gnrh gene expression was seen after a 4-hour exposure to TCDD with a 1.6-fold induction compared with vehicle control-treated GnV-3 cells (Fig.3B). Interestingly, knocking down the *Ahr* by siRNA resulted in a statistically significant 1.5-fold increase in *Gnrh* expression at 4 hours, which remained elevated at 6 hours (Fig.3C). This indicates that *AhR* is involved in regulation of *Gnrh* gene expression, even without the presence of an exogenous *AhR* ligand. In the presence of the *AhR* ligand TCDD, the time-dependent effects of TCDD on *Gnrh* expression could no longer be observed (Fig.3C), suggesting an attenuation of cyclicity in *Gnrh* expression by *AhR*. A study of Petroff et al. (2003) showed no alteration of GnRH accumulation and Gnrh promoter-reporter activity in an immortalized murine hypothalamic GT1–7 cell line after treatment with TCDD, which led the authors to conclude that dioxins might act only indirectly on the GnRH neurons. However, as mentioned above, the GT1-7 cell line lacks expression of the *AhR*, which could explain the different outcomes of our studies. Considering

the physiological role of the specific frequency of the pulsatile GnRH release and its importance in regulation of pituitary gonadotropins, any alterations in GnRH pulse frequency and/or production, may have undesirable effects on the hypothalamus-pituitary-gonadal (HPG) axis. Perturbations in circadian clock have been suggested to be associated with the promotion of some metabolic diseases and cancers (Garrett and Gasiewicz, 2006; Xu et al., 2010). Further studies on GnRH promoter activity are needed to evaluate and determine the direct involvement of *AhR* in GnRH modulation.

3.3.2. Neuropeptide Y (Npy) alteration by TCDD

Neuropeptide Y (NPY) is one of the orexigenic neuropeptides and is said to be the key generator and modulator of metabolic responses to food intake within the hypothalamus (Ramos et al., 2005). Here, we determined possible modulatory effects of AhR activation on Npy mRNA expression in GnV-3 cells. No change in constitutive *Npy* expression was observed up to 24 hours in vehicle control-treated GnV-3 cells (data not shown). TCDD time-dependently affected *Npy* mRNA levels causing a statistically significant 2.3-fold increase of expression after 6 hours (Fig.4A). This effect appears to be transient as at 24 hours it is back to control level (Fig.4A). This concurs with several *in vivo* findings that TCDD can affect orexigenic genes in rat brain. Fetissov et al. (2004) observed a significant up-regulation of *Npy* mRNA expression in the hypothalamus of rats treated with a single oral dose of TCDD (15 µg/kg). In another *in vivo* study, oral administration of TCDD (50 µg/kg) affected gene expression of a series of orexigenic neuropeptides including *Npy* in rat hypothalamus (Lindén et al., 2005).

Next, we addressed the question whether the observed changes in *Npy* gene expression after TCDD exposure are AhR–mediated in GnV-3 cells. We observed no differences in *Npy* gene expression between negative control (mock) and siRNA against *Ahr* transfected-control cells, suggesting that basal *Npy* expression is not regulated by AhR (data not shown). Nevertheless, TCDD exposure resulted in a significant induction of *Npy* mRNA expression, reaching a significant 1.7 fold *Npy* up-regulation at 24 hours (Fig.4B). Moreover, the TCDD effects were abolished in siRNA against *Ahr*- transfected cells treated with TCDD (Fig.4B). This strongly suggest the involvement of AhR in TCDD-induced *Npy* mRNA alteration. It is well known that dioxin exposure causes a dramatic reduction in food intake and a subsequent decline in body weight (Seefeld et al., 1984). Our data suggest that the effects of TCDD on hypothalamic *Npy* could play a role in the observed feeding behavior.

3.4. Neurotransmitter receptors in GnV-3 cells

GnV-3 cells cultured in differentiation medium containing doxycycline develop characteristics of adult neurons (Salvi et al., 2006). To further assess the neuroendocrine profile of GnV-3 cells, we investigated the expression of the several abundantly expressed neurotransmitter receptors in hypothalamus, *i.e.* three inotropic glutamate receptor subtypes: Kainate (*Grik2*), N-Methyl-D-aspartic acid (*NMDA,Grin1*) and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid 1 (*AMPA1, Gria1*) (Dhandapani and Brann, 2000). Also, gene expression of *Gabra*$_2$

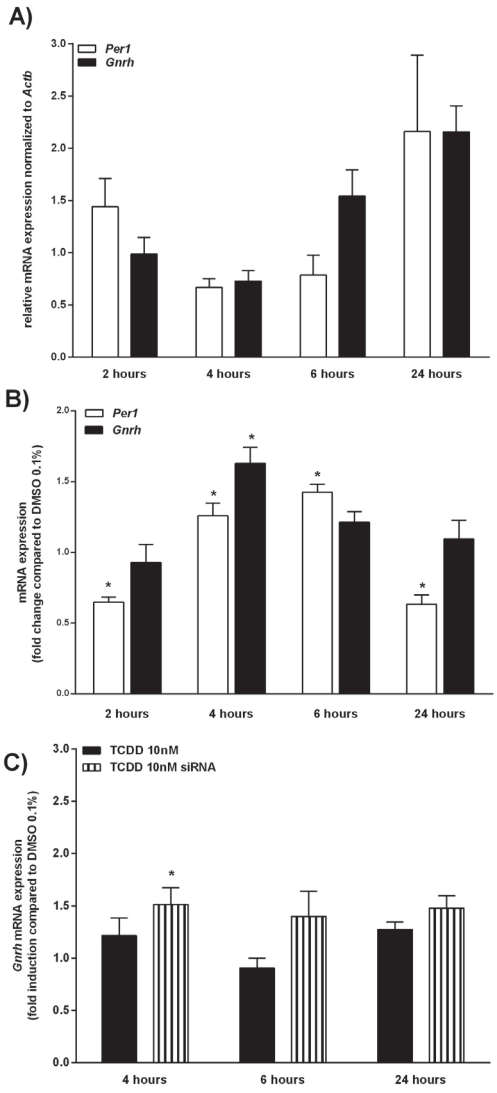

Fig. 3. A) Changes over time in mRNA expression of *Per1* (white bars) and *Gnrh* (black bars) in GnV-3 cells. Expression is shown as mean values + SEM (N=3) of relative mRNA levels expressed as ΔΔCt values of the transcript/*Actb*. B) Effect of 10 nM TCDD exposure on mRNA expression of *Per1* (white bars) and *Gnrh* (black bars) in GnV-3 cells at various time points. Expression is shown as mean values + SEM (N=3) of fold-change in expression compared with DMSO-treated cells at the same time-point. C) *Gnrh* mRNA expression upon TCDD exposure in non-transfected GnV-3 cells (black bars) or GnV-3 cells that were transfected with siRNA against *Ahr* cells (striped bars). Data are expressed as fold-change compared with expression in vehicle-control treated cells at the same time. Bars represent mean+SEM of four independent experiments that were performed in triplicate. *Significantly different from vehicle control-treated cells at the same time point, $P < 0.05$.

and Ht_{2C} receptors was studied. Using qRT-PCR, we were able to demonstrate the expression of glutaminergic, serotonergic and GABA-ergic receptor subtypes in the GnV-3 cell line (data not shown). Next, it was investigated whether AhR activation by TCDD would affect the expression of these receptors. Exposure to TCDD resulted in a significant 2.5-, 2.2- and 1.8-fold induction of $Grik_2$, Ht_{2C} and $Gabra_2$ receptor gene expression, respectively, after 4 hours (Fig.5). After 24 hours, only expression of Ht_{2C} was still significantly higher than vehicle control-treated cells. Results from *in vivo* studies indicate that TCDD impairs NMDA receptors, which are known to play an essential role in memory formation and maintenance (Kakeyama et al., 2001a, 2003b). Other studies indicated AhR-expressing GABA-ergic neurons as direct target for TCDD as TCDD was able to suppress GABA synthesis during brain development in rats (Hays et al., 2002). Our results suggest that a short time exposure to TCDD is able to disrupt the mRNA levels of important neurotransmitter receptors in GnV-3 cells. Additionally, variations within TCDD-exposed GnV-3 cells were most likely linked with the heterogenic nature of these neurons. To further analyze the functionality of the neurotransmitter profile in GnV-3 cells, we focused on 5-HT neurotransmission, which was previously shown in *in vivo* studies to be affected by TCDD (Unkila et al., 1993a,1995b).

3.5. Serotonin (5-HT) release and calcium (Ca2+) imaging studies

Serotonergic regulation of the hypothalamus has been implicated as an important component in the homeostatic control of energy balance. Additionally, 5-HT has been implicated in a variety of roles in the central nervous system including regulation of food intake and body weight. On a cellular level, 5-HT is involved in the regulation of neuropeptide secretion and intracellular calcium concentration ($[Ca^{2+}]i$) in neurons (Leibowitz et al., 1998). For that reason, we focused in our study with GnV-3 cells on the effect of AhR activation by TCDD on serotonergic signaling.

3.5.1 5-HT release

Considering the observed prolonged elevation of Ht_{2C} mRNA expression by TCDD in GnV-3 cells (Fig.5), we determined whether 5-HT secretion by GnV-3 cells was also affected by AhR activation. Using a highly sensitive ELISA, 5-HT release could be measured in culture medium of GnV-3 cells. Basal secretion levels of 5-HT were low, but detectable at 1.0 ± 0.08 pg/ml. Then, GnV-3 cells were exposed to TCDD (10 nM) and DMSO (0.1%, v/v) for 2 hours after which medium containing TCDD and vehicle was removed and 5-HT secretion was measured for 1 hour. This pre-exposure to TCDD did not significantly affect 5-HT secretion (see Supplementary data Fig.S3). Although, a slight, yet not statistically significant 1.5-fold increase in 5-HT secretion was observed between control and TCDD-exposed cells. Despite the minor effects on 5-HT release by TCDD, together with the observed increased expression of the Ht_{2C} receptor, this might result in a synergistic effect on serotonergic signaling. We believe that the elucidation of the anorexigenic action of TCDD may help understanding the biochemical mechanisms involved in the regulation of food intake and body weight, which would benefit toxicological and medical research.

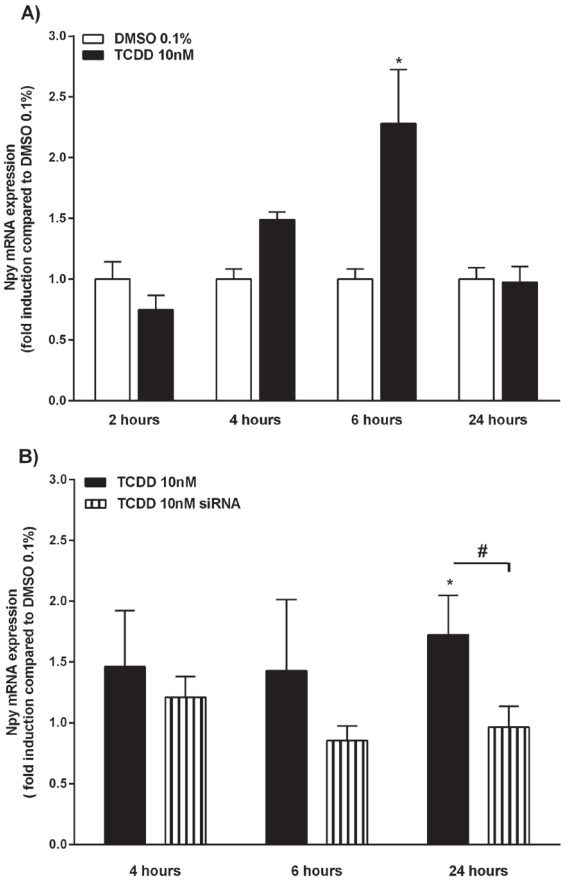

Fig. 4. A) *Npy* mRNA expression in GnV-3 cells at various time points after TCDD (10 nM) exposure. Expression is shown as mean values + SEM (N=3) of fold-change in expression compared with DMSO-treated cells at the same time-point. B) *Npy* mRNA expression upon TCDD exposure in non-transfected GnV-3 cells (black bars) or GnV-3 cells that were transfected with siRNA against *Ahr* cells (striped bars). Data are expressed as fold-change compared with expression in vehicle-control treated cells at the same time. Bars represent mean + SEM (N=4). *Significantly different from vehicle control-treated cells at the same time point, P <0.05. # Significantly different from TCDD-exposed cells.

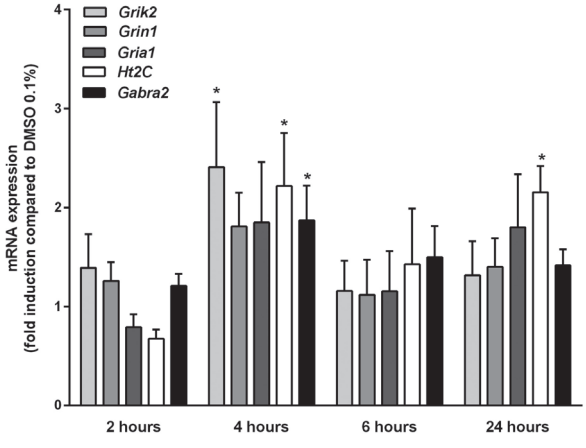

Fig. 5. Changes over time in mRNA expression of *Grik2, Grin1, Gria1, Ht_{2c}* and *$Gabra_2$* receptors in GnV-3 cells. Expression is shown as mean values+SEM (N=3) of fold-change in expression compared with DMSO-treated cells at the same time-point. *Significantly different from vehicle control-treated cells at the same time point, P <0.05.

3.5.3 Ca^{2+} imaging

To further focus on the functionality of the 5-HT neurotransmitter receptor system in GnV-3 cells, single cell fluorescent Ca^{2+} imaging studies were performed to monitor changes in $[Ca^{2+}]i$. GnV-3 cells maintain low basal $[Ca^{2+}]i$ that markedly increased upon superfusion with 100 µM 5-HT-containing saline. A subsequent stimulation with 100 µM 5-HT-containing saline following a 9-min recovery period and a 15 min exposure to 0.1% DMSO resulted in a comparable increase in $[Ca^{2+}]i$, yielding a treatment ratio (TR) of ~98% (Fig.6A and Supplementary data Table S2). Basal $[Ca^{2+}]i$ and TR were not affected by a 15 min exposure to 10 nM TCDD (Fig.6B and Supplementary data Table S2), indicating that TCDD does not affect basal and 5-HT-evoked calcium signaling. Moreover, despite the observed TCDD-induced increased expression of Ht_{2C} receptor at mRNA levels (Fig.5) and slight increase in 5-HT secretion (Fig.S3), exposure to TCDD (10 nM) for 0, 2, 6, or 24 hours did not affect basal and 5-HT evoked $[Ca^{2+}]i$ in GnV-3 cells (Fig.6C and D and Supplementary data Table S2). In line with our results, only concentrations of TCDD greater than 10 nM, which notably contributed to a marked reduction of cell viability, induced an increase in $[Ca^{2+}]i$ in N2a neuroblastoma cells (Sul et al., 2009). In contrast, in primary rat hippocampal cells, TCDD induced an increase in $[Ca^{2+}]i$ already at 10 nM, though more pronounced effects were observed following exposure to TCDD up to 100 nM (Hanneman et al., 1996). As observed recently by Tung et al. (2012), *in vitro* serotonergic stimulation of hypothalamic mHypoA-2/30 adult neurons evoked a significant release of intracellular endothelial reticulum Ca^{2+} stores. This effect was partially mediated by the 1B receptor subtype of 5-HT (Ht_{1B}) through activation of phospholipase-protein kinase C pathway. Further research on the presence of different 5-HT receptor subtypes is needed to clarify our findings.

4. Conclusion

This study was designed to determine the presence of a functional AhR signaling pathway in the novel hypothalamic GnV-3 cell line. Here, we showed that GnV-3 cells display a functional AhR system that can be activated by the potent AhR agonist TCDD in a time-dependent manner. Our study also revealed that GnV-3 cells follow an approximate 24-hour circadian rhythm, which exhibits a similar pattern as *Gnrh* mRNA. The activation of the AhR by TCDD indicated a number of AhR-dependent pathways that are associated with the circadian rhythm and food intake control, but not with 5-HT receptor regulation. Despite the limitations inherently arising from an *in vitro* cell system, such as the absence of extrinsic non-neuronal and hormonal signals that can create an *in vivo* relevant micro-environment, our findings demonstrate that the GnV-3 cell line can be utilized as an additional tool for future screening of the effects of AhR-binding ligands, including other halogenated compounds or natural compounds, to increase a mechanistic understanding of the AhR-mediated neuroendocrine mechanisms of action in the hypothalamus.

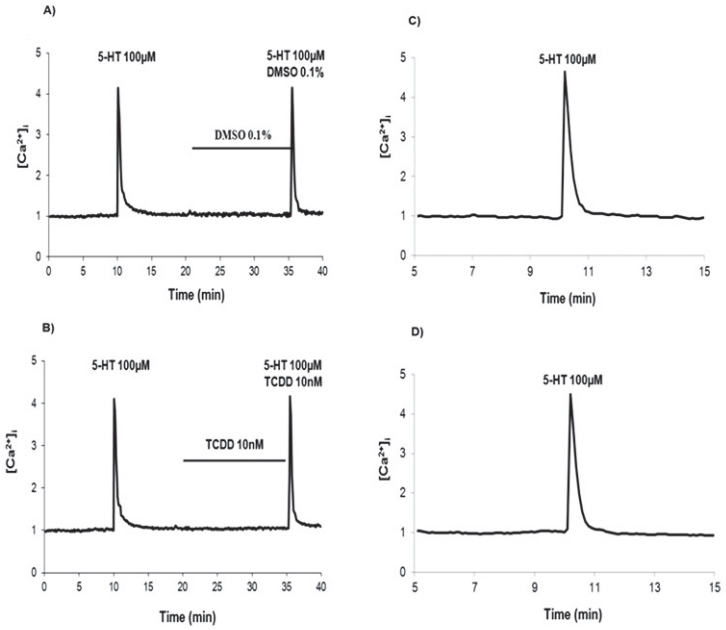

Fig. 6. Example recordings of basal and 5-HT-evoked increases in $[Ca^{2+}]i$ in GnV-3 cells exposed for 15 min to A) 0.1% DMSO or B) TCDD (10 nM). Lines show the time and duration of DMSO (0.1%) or TCDD (10 nM) exposure. 5-HT-evoked increases in $[Ca^{2+}]i$ in GnV-3 cells that have been pre-treated for 24 hours with C) DMSO (0.1% v/v) or D) TCDD (10 nM).

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Supplementary data

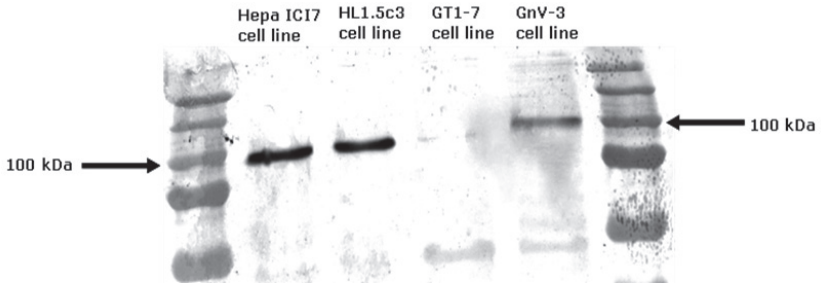

Fig. S1. Western blot analysis of AhR protein expression in GnV-3 cells growing in proliferation medium. Liver murine cell lines Hepa 1C17 and HL1.5c3 were used as positive control for presence of AhR; GT1-7 cell line is a widely-used murine hypothalamic cell line that is known to lack AhR expression.

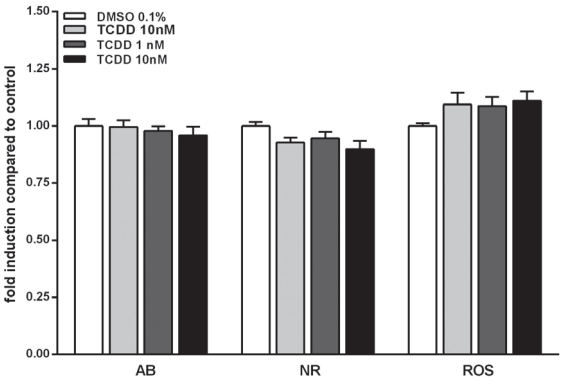

Fig. S2. Cytotoxicity tests in GnV-3 cells. Alamar Blue (AB), Neutral Red (NR) and Reactive Oxygen Species (ROS) assays were performed after a 24-hour exposure of GnV-3 cells to DMSO (0.1% v/v) or TCDD (0.1, 1 or 10 nM). Data are expressed as % compared with expression in vehicle (DMSO 0.1%)-control treated cells. The experiments were performed in triplicate and repeated three times (N=3). $P < 0.05$ was considered statistically significant.

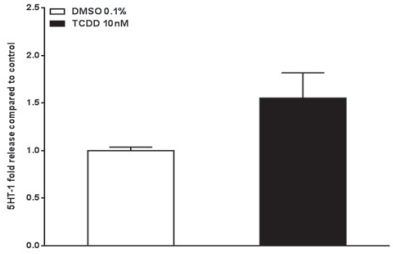

Fig. S3. Serotonin (5-HT) release by GnV-3 cells. After a 2 hour-pretreatment with TCDD (10 nM), medium was replaced with medium containing either DMSO (0.1%; white bar) or TCDD (10 nM; black bar). After 1 hour, 5-HT levels were determined by ELISA. The experiments were performed in triplicate and repeated two times (N=2). Data are expressed as fold-change compared with expression in vehicle (DMSO 0.1%)-control treated cells and were analysed using an un-paired Student's t-test. $P < 0.05$ was considered statistically significant.

Table S1. Primer sequences for gene expression analysis using qRT-PCR with SYBR green quantification. * Actb was used as reference gene.

Gene name (accession nr.)	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product [bp]
Actin, beta (*Actb*)* NM_031144.2	AGCGTGGCTACAGCTTCACC	AAGTCTAGGGCAACATAGCACAGC	85
Aryl hydrocarbon receptor (*Ahr*) NM_013149.2	TGGCTGTGATGCCAAAGGGCAG	AGCATGTCAGCGGCGTGGAT	99
Aryl-hydrocarbon receptor repressor(*Ahrr*) NM_001024285.1	CCCCAAGGGGACTTCAGGGGAC	TGCTCCAGTCCAGGTGCCTCA	146
Cytochrome P450, family 1, subfamily a, polypeptide 1 (*Cyp1a1*) NM_012540.2	TCACCATCCCCCACAGCACCA	TCAGGCCGGAACTCGTTTGGA	139
Period homolog 1 (Per1) NM_001034125	GTCCAGGGATGCAGCGTCT	GAGAACCGTGGCTGTTGTTTC	78
Gonadotropin-releasing hormone 1 (*Gnrh*) NM_012767	GGCAAGGAGGAGGATCAAA	CCAGTGCATTACATCTTCTTCTG	142
Glutamate receptor, ionotropic, (*Gria1, AMPA 1*) NM_031608.1	GAAGGGAGGGGAGGGAAGACCAA	GCACCCACAACCGCACCTAGAA	126
Glutamate receptor, ionotropic, N-methyl D-aspartate 1 (*Grin1*) NM_017010.1	CAAAGACACGAGCACCGGGGG	CTCAATAGCGCGTCGCGGCA	79
Glutamate receptor, ionotropic, kainate 2 (*Grik2*) NM_019309.2	CTTGCTCCTTGGCGCAGTGAGAG	TCCGGGCTTGGACGAGAAGGAA	147
Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 2 (*Gabra2*) NM_001135779.1	TGTAGCCGTTGCCAACTACGCC	CGGCTGGCTTGTTCTCTGGCTT	113
Neuropeptide Y (*Npy*) NM_012614.1	TGGGCATTCTGGCTGAGGGGT	CTGGCCATGTCCTCTGCTGGC	76
Aryl hydrocarbon receptor nuclear translocator 1 (*Arnt1*) NM_012780.1	GCGACGGTCAGGGCTGGATT	TCATCCGACCTGGCAAACCGC	111
Aryl hydrocarbon receptor nuclear translocator 2 (*Arnt2*) NM_012781.3	GCGGCGATCCGGAATGGACT	GCCTGCGCCGCTCAATCTCA	92
Htr2c 5-hydroxytryptamine (serotonin) receptor 2C (*Htr2c*) NM_012765.3	CCATCATGCACCTCTGCGCCA	CCGCGAATTGAACCGGCTATGC	83

Table S2. The effects of TCDD exposure on basal and 5-HT evoked changes in internal calcium levels [Ca^{2+}]i in GnV-3 cells. Exposure of GnV-3cells for 15 min to TCDD (10 nM) does not affect the TR (see materials and methods for details) compared to cells exposed to 0.1% DMSO. GnV-3 cells exposed to TCDD (10 nM) for 0-24 hours have comparable basal levels of [Ca^{2+}]i as well as comparable levels of 5-HT-evoked [Ca^{2+}]i. Data were presented as mean+SEM of n cells obtained from N independent experiments.

	TR		Basal $[Ca^{2+}]_i$				5-HT-evoked $[Ca^{2+}]_i$			
	DMSO (0.1%)	TCDD (10 nM)	0h	2h	6h	24h	0h	2h	6h	24h
Av (%) + SEM	97,9 + 4,3	95,0 + 6,7	0,71+ 0,005	0,73+ 0,0065	0,74+ 0,0065	0,71+ 0,0053	4,71+ 0,07	4,76+ 0,071	4,37+ 0,081	4,74+ 0,068
n (N)	68 (5)	44 (4)	94 (6)	128 (7)	72 (5)	104 (5)	87 (6)	109 (7)	75 (5)	99 (5)
	15 min exposure		cells pre-treated with TCDD (10 nM) for 0, 2, 6 and 24 hours				cells pre-treated with TCDD (10 nM) for 0, 2, 6 and 24 hours			

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CHAPTER 4

Excessive levels of phytoestrogens can modulate ovarian steroidogenesis and tumor progression in KGN human granulosa-derived tumor cells.

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Abstract

Phytoestrogens are plant-derived estrogen-like compounds that are increasingly used for their suggested health promoting properties, even by healthy, young women. However, scientific concerns exist regarding potential adverse effects on female reproduction. In this study, naringenin (NAR), 8-prenylnaringenin (8-PN), genistein (GEN), coumestrol (COU), quercetin (QUE) and resveratrol (RSV) up-regulated steroidogenic acute regulatory protein (*StaR*) mRNA levels in KGN human granulosa-like tumor cells. Most of the phytoestrogens tested also increased *CYP19A1* (aromatase) mRNA levels via activation of ovary-specific *I.3* and *II* promoters. Yet, only NAR (3 and 10 µM), COU (10 and 30 µM) and QUE (10 µM) also statistically significantly induced aromatase activity in KGN cells after 24 hours. 8-PN, aromatase inhibitor letrozole and estrogen receptor antagonist ICI 182,780 concentration-dependently inhibited aromatase activity with IC_{50} values of 8 nM, 10 nM and 72 nM, respectively. Co-exposure with ICI 182,780 (0.1µM) statistically significantly attenuated the induction of aromatase activity by QUE and COU, but not NAR. Cell cycle status and proliferation of KGN cells were not affected by any of the phytoestrogens tested. Nonetheless, the migration of KGN cells was significantly reduced with approximately 30% by COU, RSV and QUE and 46% by GEN at 10 µM, but not NAR and 8-PN. Our results indicate that phytoestrogens can affect various pathways in granulosa-like cells *in vitro* at concentrations that can be found in plasma upon supplement intake. This implies that phytoestrogens may interfere with ovarian function and caution is in place regarding the use of supplements with high contents of phytoestrogens.

1. Introduction

Phytoestrogens are naturally occurring plant compounds that are omnipresent in our daily diet. Over the past years, phytoestrogens have attracted much attention due to their estrogenic or anti-estrogenic properties and their potential use as alternatives for hormone replacement therapy in postmenopausal women. In addition, supplements that contain high levels of phytoestrogens are commercially available for breast enhancement and relieve of menstrual complaints. Phytoestrogens are diverse plant-derived group of compounds and they consist of several groups of different chemical classes classified according to their chemical structure including flavanoids, coumestans and lignans. Most flavanoids are present in plants in the conjugated glycoside forms and can be readily hydrolysed by gastrointestinal bacteria to biologically active aglycones [1]. There is considerable interest in whether human exposure to phytoestrogens has any health risks or benefits due to the increase of nutritional and pharmaceutical use of dietary phytoestrogenic compounds [2; 3]. Epidemiologic evidence mainly based on Asian population studies, supports a protective effect of high phytoestrogen diets to reduce the incidence of certain hormone-responsive cancers, such as breast and prostate cancer. Contrary, there have been concerns that phytoestrogens, through their estrogenic properties, may increase the risk of recurrence or stimulate the growth of existing tumors. In addition, numerous *in vivo* and *in vitro* studies have demonstrated altered ovarian function and changes in the developing female reproductive system following exposure to phytoestrogens in laboratory animal studies [4, 5; 6]. Phytoestrogens were able to affect female reproductive function by modulating the female cycles that in turn resulted in infertility in animals [7; 8] and humans [9; 10]. In humans, the prevalence of precocious puberty was significantly higher in Korean girls with high serum isoflavone levels [11]. Despite the numerous studies, the molecular *mechanisms underlying the adverse effects of phytoestrogens on ovarian function still remain elusive.*

It is well known that phytoestrogens may disrupt endocrine-dependent processes by acting as estrogen receptor (ER) agonists or antagonist due to their bi-phenolic structure required for ligand-receptor association. Phytoestrogens can bind weakly to ERs, typically with affinities that are 1000 times less than that of 17β-estradiol (E2) [12]. ERβ receptor is a classical steroid receptor predominantly expressed in granulosa cells. In contrast, ERα protein is expressed at low levels in granulosa cells [13]. Several phytoestrogens are selective estrogen receptor modulators that have greater affinity for ERβ than ERα [14]. In addition to classical estrogen receptors, phytoestrogens were shown to be ligands for the non-classical estrogen receptor G-protein coupled protein receptor 1 GPER1 [15]. Moreover, it has become clear that phytoestrogens can exert endocrine disrupting properties by inhibiting key steroidogenic enzymes. During puberty, E2 that is synthesized and secreted by granulosa cells in the ovaries, modulates the structure and function of female estrogen-sensitive tissues and contributes to maintaining a proper menstrual cycle pattern and female sexual behavior. Ovarian steroidogenesis is initiated by the delivery of cholesterol from cytosol into the mitochondria by the steroidogenic acute regulatory protein (StAR)

[16; 17]. The final step in estrogen synthesis is catalyzed by aromatase (*CYP19A1*), which converts androgens into estrogens. Human CYP19A1 comprises of ten exons including exons II–X that encode the aromatase protein and 3'-untranslated region of the mRNA. Alternative first exons encode unique 5'-untranslated regions of the aromatase mRNA transcripts in different estrogen-producing tissues [18; 19]. Aromatase transcripts in gonads, brain, adipose and placenta contain different first exons (II, If, I.4/I.3 and I.1, respectively) and the expression of CYP19A1 in each of these organs is controlled by alternatively spliced tissue-specific promoters regulated by distinct signaling pathways in a hormone-specific manner [19; 20; 21; 22; 23]. In ovarian granulosa cells, aromatase expression is FSH-driven and is regulated via the ovary-specific PII promoter [21]. Many studies with various models have shown the inhibitory effects of phytoestrogens on aromatase activity [24; 25; 26; 27; 28; 29], while other phytoestrogens induce aromatase activity [30; 31].

The aim of our work was to study the effects of several potent phytoestrogens that are frequently used in dietary supplements as possible modulators of ovarian function and cellular behavior *in vitro*. It should be noted that many phytoestrogens are omnipresent in our daily diet, albeit at much lower concentrations than can be found in dietary supplements. The data from these studies were used to assess the potential risk for ovarian dysfunction in humans upon high intake levels of phytoestrogens including naringenin (NAR), 8-prenylnaringenin (8-PN), genistein (GEN), coumestrol (COU), quercetin (QUE) and resveratrol (RSV) (for chemical structures see supplementary data Fig.S1). These phytoestrogens were selected for their previously reported induction (GEN and QUE) [30; 31] or inhibition (NAR and 8-PN) [29; 30; 31] and/or their reported effects on ovarian tumor cell behavior (RSV, GEN, QUE, COU) [32; 33; 34]. In our study, we used the KGN granulosa-like tumor cell line of human origin. KGN cells were previously shown to maintain many of the physiological features of normal human granulosa cells, including steroidogenesis [35] and secretion of estrogens [36; 37]. We show here that KGN cells display the ovarian-specific PII/I.3-driven aromatase expression, similarly to normal granulosa cells surrounding the preovulatory follicle. KGN cells have also been reported to express both ERs [38]. Moreover, several studies have demonstrated that KGN cells respond similarly to primary human granulosa cells upon stimulation with e.g. FSH [39; 40; 41]. Therefore, this cell line is an excellent and applicable *in vitro* model to study effects on human granulosa cell functioning. Here, the action of the selected phytoestrogens on ovarian steroidogenic enzymes such as StAR and CYP19A1 and its promoter-specific expression was investigated. Phytoestrogens have also been shown to affect a wide array of intracellular signaling mechanisms that are important for regulating cell cycle progression. Therefore, we investigated to which extent phytoestrogens influence the metastatic properties of KGN cells by performing a wound healing assay. Finally, expression of several important genes involved in cell progression and/or death, were studied. We chose to study gene expression of *VEGF*, a critical inducer of tumor angiogenesis and *SIRT1*, regulator of cellular lifespan and tumor promoter in mammary epithelial cells [32; 42]. Moreover, gene expression of *MMP9,* a prerequisite for enhanced cell migration, and *CADHERIN E*, adhesion-activated signaling receptor, were evaluated. The data from these *in vitro* studies

were compared with reported human plasma levels to assess the potential risk for ovarian dysfunction in humans upon high intake levels of phytoestrogens.

2. Material and methods

2.1 Cell culture

The KGN granulosa-like tumor cells (kindly provided by Riken Biosource Center, Tsukuba, Japan) were cultured in supplemented with 10% FBS (Invitrogen-Gibco) phenol red-free DMEM/F12 (Invitrogen-Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen-Gibco) in an atmosphere of 5% CO_2/ 95% air at 37°C. KGN cells were subcultured 1:2 once a week. For all experimental conditions, media were replaced with 5% steroid free Serum dextran/charcoal (Hyclone; Invitrogen-Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen-Gibco) and KGN cells were grown for 4 next days before treatments.

2.2 Chemicals and reagents

Estradiol (E2), NAR, 8-PN and dexamethasone (DEX) were dissolved in ethanol. GEN, COU, QUE, RSV, ICI 182,780 and Prostaglandin E2 (PGE2) were dissolved in dimethyl sulfoxide (DMSO). 5-bromo-2-deoxyuridine (BrdU) was dissolved in Phosphate buffered saline (PBS). All compounds were purchased from Sigma-Aldrich Co. (Zwijndrecht, The Netherlands), unless indicated otherwise. BrdU was purchased from Euro-diagnostics bv, Apeldoorn, the Netherlands. The concentration of each solvent was set as 0.1% of the culture medium.

2.3 Cytotoxicity assay

Cell viability was determined according to Denizot and Lang (1986) [43] by measuring the capacity of KGN cells to reduce MTT (3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan by the mitochondrial enzyme succinate dehydrogenase. Briefly, KGN cells (0,1 X 10^6) were plated in 24 well plates in assay medium and then incubated for 1.5 hour with MTT at 37°C. After that time, the formed blue colored formazan was extracted by adding 1 mL of isopropanol at room temperature (RT). Absorbance was measured spectrophotometrically at an absorbance wavelength of 595 nm (POLARstar Galaxy, BMG Labtech GmbH, Ortenberg, Germany).

2.4 Quantitative real time polymerase chain reaction (RT-PCR)

Gene expression studies were performed in KGN cells. Briefly, KGN cells (2.5 X 10^5 cells) were seeded in 12-well plates in assay phenol red-free DMEM/F12 medium. Cells were exposed to NAR and 8-PN at 3 µM ; GEN, COU, QUE and RSV at 10 µM for 24 hours. PGE2 (0.1 µM), a potent stimulator of steroidogenic genes such as StAR and CYP19A1 of ovarian-related aromatase [44] was used as a positive control for aromatase induction. Total RNA was harvested from the KGN cells by phenol–chloroform extraction using RNA Instapure (Eurogenetic, Liege, Belgium). Purity and concentration of the RNA samples were determined spectrophotometrically at an

absorbance wavelength of 260/280nm and 230/260 nm. Complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis Kit, according to the manufacturer's instructions (Biorad, Veenendaal, the Netherlands). Obtained cDNA was diluted 10 times and stored at -20 °C until further analysis. The qRT-PCR was performed using CFX Manager (Biorad, Veenendaal, the Netherlands). PCR reaction was initiated by heating at 95 °C for 3 minutes, then followed by 40 cycles with denaturation at 95 °C for 15 seconds, annealing/extension at 60 °C for 45 seconds. After each run a melt curve was performed to ensure that primer-dimers and other non-specific products were omitted. A negative control sample (non-RT) was included in each run. The primers for *β-Actin*, I.3, PII, I.4, Vascular Endothelial Growth Factor (*VEGF*) have been described previously [45; 46]. All primer pairs used in the qRT-PCRs are listed in Table 1.The primers, were designed using Primer-BLAST of National Center for Biotechnology Information (NCBI) and then checked with BLAST (nucleotide nonredundant database) to confirm specificity. The *β-Actin* gene expression was not affected by tested phytoestrogens, thus it was used as housekeeping gene in the present study.

2.5 Aromatase (*CYP19A1*) activity

CYP19A1 (aromatase) activity was determined in KGN cells after a 24-hour exposure using the tritiated water-release method of Lephart and Simpson (1991) [47] with minor modification by Sanderson et al. (2001) [48]. Briefly, KGN cells (0,1 X 10^6) were seeded onto 24 well plates in assay medium. CYP19A1 activity was measured as the amount of tritiated water formed after the conversion of the CYP19A1 enzyme's substrate [1β–^{3}H)-androstenedione. PGE2 (0.1 μM), was used as a positive control for aromatase catalytic activation.

2.6 Protein extraction and Western Blot analysis

KGN cells (6 x 10^6) were washed once with warm PBS, then trypsinized. Cells were centrifuged and 250 μL of PBS was added to each cell pellet. Cells were lysed using adopted freeze-thaw lysis method using liquid-Nitrogen and 37°C water bath by alternatively incubating at least 3 times The total protein concentration of lysates was quantified using he Lowry assay [49]. 30 μg of cell protein and protein standards were fractionated by SDS-PAGE (10%), electrophoretically transferred onto polyvinylidene fluoride (pvdf) membrane and probed for 1 hour at RT with a primary rabbit monoclonal antibody recommended for detection of human CYP19A1, dilution factor 1:1000 (Abcam). After washing, the membranes were incubated for another 1 hour at RT with a secondary polyclonal goat anti-rabbit antibody conjugated to the enzyme horseradish peroxidase (HRP), dilution factor 1:1000 (Abcam). Protein bands were visualized by chemiluminescence using Bio-Rad Clarity™ western ECL substrate kit. Precision Plus Protein™ Standards were used at the 10-250 kDa range as the markers (Bio-Rad Laboratories, Inc.).

2.7 Wound healing assay

The wound healing assay was described previously [50] and was used to determine whether KGN cell motility could be affected by diverse phytoestrogens. KGN cells (0,7

X 10^6) were cultured in 12-well cell plates until confluency. Wounds were made by scratching the cellular layer with a 100 µl pipette tip in the middle of the well. After washing away the cell debris, assay medium with tested phytoestrogens was added to the culture. Zero hour pictures (0 hour-control) were taken for each wound with an Olympus U-CMAD3 camera. Cells were incubated for 8 and 24 hours and then another picture for each wound was taken. The wound area was measured with an Image J 1.47c software (National Institutes of Health, USA).

2.8 BRdU staining

KGN cells (0,7 X 10^6) were cultured in 12-well cell plates until confluent. BrdU (10 µM) was added to culture medium for 1 hour. When added to culture medium, BrdU (10 µM) is incorporated into the DNA of cells that are in the S-phase of the cell cycle [51]. Then, KGN cells were fixed in ice-cold methanol for 20 minutes at 37 °C. Immunocytochemical detection of BrDU labled DNA was performed using undiluted anti-BrDU and peroxidase-conjugated Rabbit-anti-mouse Ig (RAM-PO, dilution factor: 1:40) for 90 and 60 minutes, respectively.

2.9 Cell cycle analysis

Cell cycle analysis was determined according to protocol described by Sangjun et al, 2009 [52]. KGN cells (0,25 X 10^6) were plated onto 12-well plates and further cultured with phytoestrogens for 24 hours. Cells were then trypsinized, fixed and permeabilized with 70% ethanol for at least 30 minutes at 4°C. KGN cells were then labeled with propidium iodide for 10 minutes at room temperature (RT). The samples were used for flow cytometry (FACS, Calibur, Becton Dickinson) to determine the cell cycle distribution of the KGN cells. The flow cytometry data was analyzed using Flowjoj software (Tree Star, Inc, USA).

2.10 Data analysis and statistics

All experiments were performed at least three times and within each independent experiment each concentration was tested in duplicate (aromatase activity) or triplicate. The results are displayed as the mean of replicates of each experiment with standard deviation (SD). Statistical analysis of difference of the means between vehicle-treated control (either Ethanol (0.1%) or DMSO (0.1%) or combination of both) -treated cells was determined using a two-tailed unpaired Students' t-test or a one-way ANOVA and post-hoc Dunnett's test. Calculations are performed using GraphPad Prism 6.01 (GraphPad Software Inc. San Diego, USA). Differences were considered statistically significant with $P < 0.05$.

3. Results

3.1. MTT cytotoxicity assay

None of the tested compounds was cytotoxic at the highest concentration tested, except for GEN (30 µM) and COU (30 µM) (supplementary data Fig.S2B). At the highest concentrations tested, GEN and COU reduced cell viability by 20 and 15%,

respectively when compared to vehicle-treated control cells. In the subsequent experiments with KGN cells, non-cytotoxic concentrations of phytoestrogens were used.

3.2 Modulation of aromatase in KGN cells

3.2.1 Aromatase (CYP19A1) gene expression

Aromatase (*CYP19A1*) expressed in granulosa cells surrounding the developing oocyte in the ovary, is responsible for conversion of androgens into estrogens. *CYP19A1* mRNA levels in KGN cells statistically significantly increased by 2.3, 1.9 and 3.2 -fold after a 24-hour exposure to 8-PN (3 µM), GEN (10 µM) and QUE (10 µM). No changes in *CYP19A1* mRNA levels were seen in NAR (3 µM), COU (10 µM) and RSV (10 µM) treated-KGN cells (Fig.1A). A 24-hour exposure to PGE2 (0.1 µM) increased *CYP19A1* mRNA by approximately 7-fold compared with vehicle-treated control cells (Fig.1A). Using qRT-PCR, we showed that aromatase expression in KGN cells was promoter II (PII) and I.3-driven, as expected based on its ovarian origin. Expression of both *PII*- and *I.3*-driven CYP19A1 was statistically significantly up-regulated by PGE2 (0.1 µM) after 24 hours and increased 3.5- and 4-fold for *I.3* and *PII*, respectively (Fig.1B). Neither promoter *I.4* nor I.1-driven *CYP19A1* expression was detected in KGN cells (data not shown). Also, DEX (0.1 µM), a potent inducer of promoter *I.4*-driven transcription of *CYP19A1* did not induce aromatase gene expression and activity (data not shown). Based on these data, we tested the effects of phytoestrogens only on *PII/1.3*-driven *CYP19A1* gene expression. Among the phytoestrogens tested, QUE (10 µM) was the most potent activator of *PI.3* and *PII*-driven *CYP19A1* (3.5- and 3-fold induction, respectively). Also 8-PN and GEN statistically significantly up-regulated *PII/PI.3*-driven *CYP19A1* expression. 8-PN (3 µM) significantly up-regulated *CYP19A1* via promoters 1.3 and II by 1.8- and 2-fold, respectively. GEN (10 µM) had more potent effects towards activation of *PI.3* promoter than *PII*, with a statistically significantly increase of I.3 promoter-driven *CYP19A1* mRNA to about 2-fold compared with vehicle-treated control cells. Neither NAR nor COU and RSV affected promoter-specific mRNA expression of *CYP19A1*. Taken together, the results demonstrated stimulatory effects of diverse phytoestrogens on promoter *I.3* and *II*-driven mRNA of *CYP19A1* in KGN cells.

3.2.3 Aromatase CYP19A1 enzyme activity and expression

The known inducer of ovarian-type aromatase PGE2 (0.1µM) statistically significantly induced aromatase activity up to 315% compared with vehicle-treated control cells (data not shown). In contrast, the known specific *CYP19A1* enzyme inhibitor letrozole concentration-dependently inhibited CYP19A1 activity in KGN cells (IC_{50} value of 10 nM; Fig.2A). Of all tested phytoestrogens, only 8-PN inhibited aromatase activity (IC_{50} value of 8 nM; Fig.2B). COU and NAR concentration-dependently increased aromatase activity to approximately 155% at non-cytotoxic concentrations of 10 µM when compared to vehicle-treated control cells (Fig.2B,C). Apparent EC50 values for induction of aromatase activity were 10 and 1.3 µM for COU and NAR, respectively. QUE was the most potent CYP19A1 activator with an EC_{50} value of 4.7 µM and a maximum induction of 210% at 10 µM. Notably, there was a sharp and significant

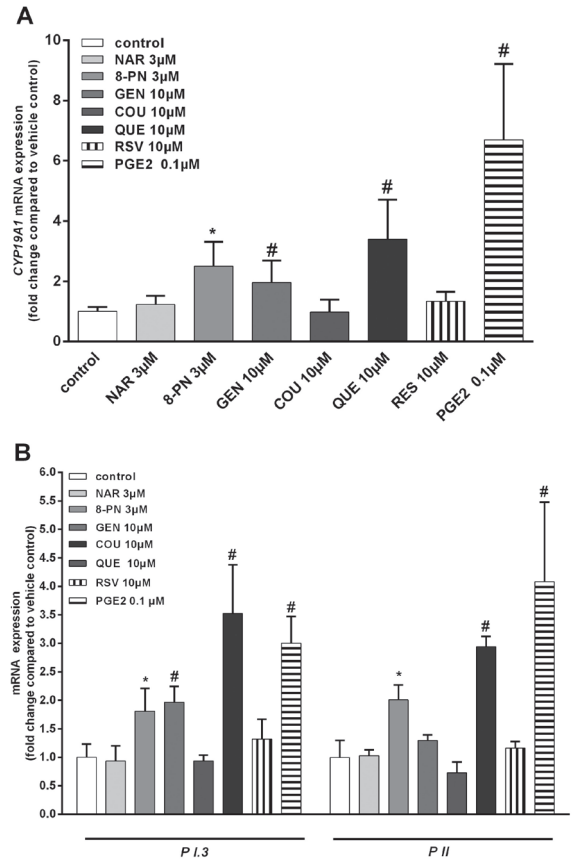

Fig.1 Gene expression in KGN cells after a 24-hour exposure to various phytoestrogens. (A) *CYP19A1* mRNA levels. Significant up-regulation of CYP19A1 mRNA after exposure to 8-PN (3μM), GEN (10 μM) and QUE (10 μM). PGE2 (0.1 μM) was used as a positive control.

(B) Promoter *P1.3*- and *PII*-specific expression of CYP19A1 mRNA. Significant up-regulation of P1.3 and PII –driven CYP19A1 mRNA after exposure to 8-PN (3μM), GEN (10 μM) and QUE (10 μM). PGE2 (0.1 μM) was used as a positive control. Data are expressed as fold-change compared with expression in vehicle-control treated cells. Bars represent mean+SD of three independent experiments that were performed in triplicate (N=3). * and # significantly from relevant solvent vehicle control Ethanol (0.1%) or DMSO (0.1%) -treated cells, respectively. *P* <0.05.

decline in aromatase activity after QUE exposure at 30 μM (Fig.2C). This decline was not due to cytotoxicity (supplementary data FigS.2B). The phytoestrogens GEN and RSV did not affect CYP19A1 activity at any of the tested concentrations (Fig.2C). Because of the differential effects of phytoestrogens on CYP19A1 gene expression and aromatase activity, CYP19A1 protein expression was determined by Western Blot. A slight increase in protein level of CYP19A1 was observed by PGE2 (0.1 μM) and COU (10 μM)-treated KGN cells after 24 hours. However, no changes at CYP19A1 protein level were observed after 24-hour exposure to 8-PN (3 μM), GEN (10 μM) and QUE (10 μM) (supplementary data Fig.S3).

Phytoestrogens are suggested to exert their effects mainly via estrogen receptors. Therefore, we studied the potential involvement of estrogen receptor α (ERα) in the activation of aromatase by non-cytotoxic concentrations of NAR, COU and QUE. Exposure to E2 (0.001 – 0.1 μM) did not have a statistically significant effect on CYP19A1 activity (data not shown). However, the known pure ERα antagonist ICI 182,780 concentration-dependently reduced aromatase activity (IC_{50} value of 72 nM; Fig.2A). Moreover, co-exposure with ICI 182,780 (0.1 μM) and COU (10 μM) or QUE (10 μM), but not NAR (3 μM), statistically significantly decreased aromatase activity in KGN cells when compared to COU or QUE exposed cells alone (Fig.3).

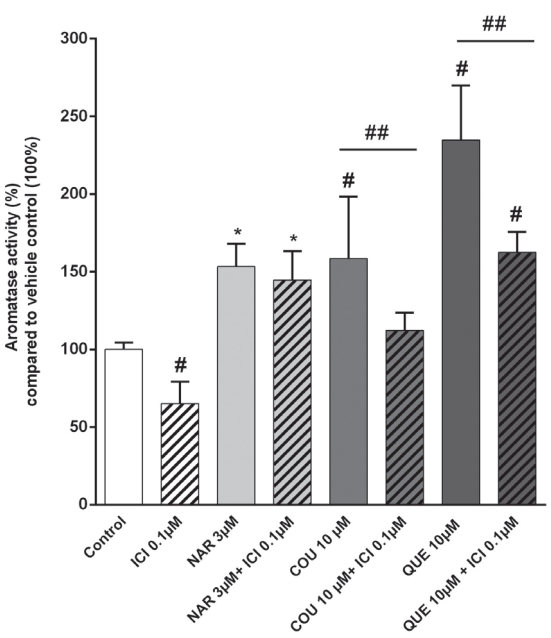

Fig.3 Aromatase activity in KGN cells exposed to ICI (0.1 µM), NAR (3 µM), COU (10 µM) and QUE (10 µM). Striped bars present a co-exposure of ICI (0.1 µM) with tested phytoestrogens. Data are expressed as % of aromatase activity compared with 100% of aromatase activity in vehicle-control treated cells. The experiments were performed in duplicates and repeated three or four times (N=3; N=4). * or # Significantly different from Ethanol (0.1%) or DMSO (0.1%) control-treated cells, respectively. ## significantly different from COU (10 µM) or QUE (10 µM)-exposed cells. $P < 0.05$.

3.3 Steroidogenic acute regulatory protein mRNA levels are up-regulated by diverse phytoestrogens

Phytoestrogen-mediated effects on StAR mRNA expression were determined in KGN cells. Results showed that *StAR* mRNA levels were statistically significantly up-regulated after a 24 hour-exposure to 8-PN (2.3-fold), 10 µM COU (1.9 fold),10 µM QUE (2.2-fold), and 10 µM RSV (2-fold) (Fig.4). Also 0.1 µM PGE2 up-regulated *StAR* mRNA by approximately 2.5-fold (Fig.4).

3.4. Phytoestrogens do not impair cell cycle but affect cell migration

A wound healing assay was performed to determine the effect of phytoestrogens on KGN cell migration. To determine the migratory properties of KGN cells, wound healing was assessed at 0, 8 and 24 hours after inflicting the wound. In vehicle-treated KGN cells, the wound area was 89% ± 6 and 40%± 2 after 8 and 24 hours, respectively, compared with the initial wound area. Based on this, a 24 hour-time point was chosen to evaluate possible inhibition or induction of phytoestrogens on KGN cell migration. The phytoestrogens tested decreased migration of KGN cells after a 24 hour- exposure (Fig.5A,B). GEN (10 µM) exerted the most pronounced effect on cell mobility and statistically significantly inhibited migration of KGN cells to 46% compared with vehicle-treated control cells. The phytoestrogens COU (10 µM), QUE (10 µM) and RSV (10 µM), but not NAR (3 µM) and 8-PN (3 µM), decreased cell migration to about 25-30% compared to vehicle-treated control cells after 24 hours (Fig.5A,B). To ensure that the observed changes on migration were not due

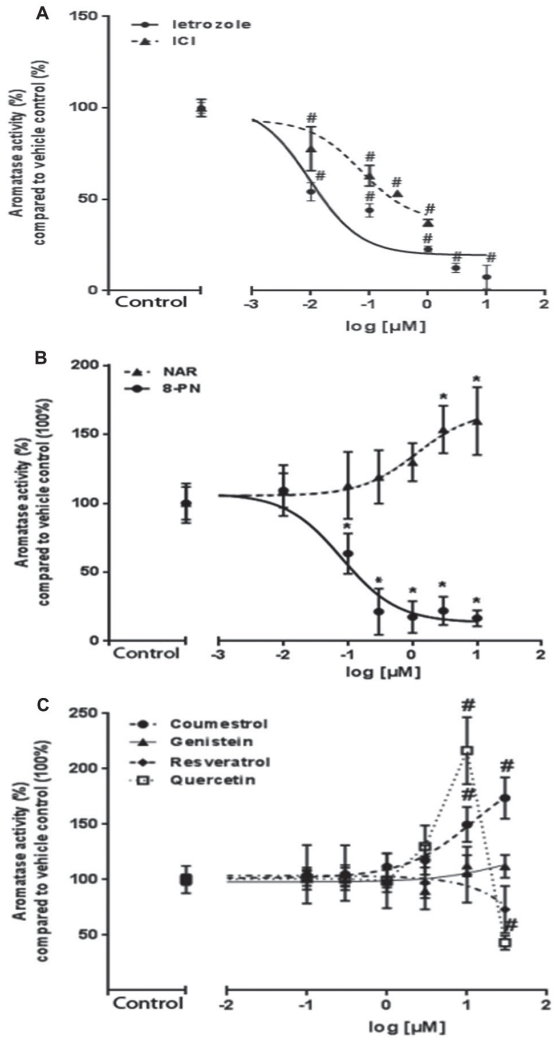

Fig. 2 Aromatase activity in KGN cells. (A) Concentration-dependent inhibition curve of Letrozole and ICI 182,780. (B) 24 hour- exposure to different concentrations of NAR and 8-PN. (C) Effects of COU, GEN, RSV and QUE at diverse concentrations on aromatase activity. Data are expressed as % of aromatase activity compared with 100% of aromatase activity in vehicle control treated cells. The experiments were performed in duplicates and repeated three or four times (N=3; N=4). * and/or # significantly different from relevant solvent vehicle control Ethanol (0.1%) or DMSO (0.1%) -treated cells, respectively. $P < 0.05$.

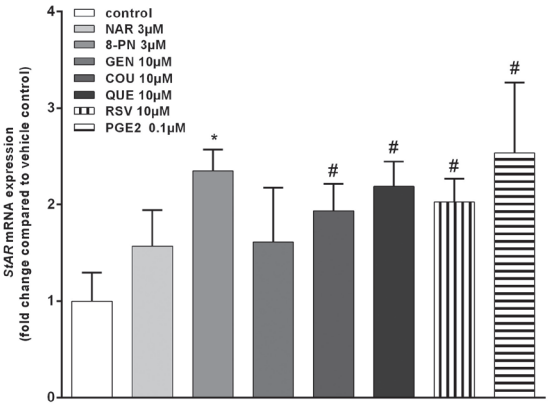

Fig. 4 *StAR* mRNA expression in KGN cells after a 24-hour exposure to diverse phytoestrogens. Data are expressed as fold-change compared with expression in vehicle-control treated cells. Bars represent mean+SD of three independent experiments that were performed in triplicate (N=3). * and # significantly different from relevant solvent vehicle control Ethanol (0.1%) or DMSO (0.1%) -treated cells, respectively. P <0.05.

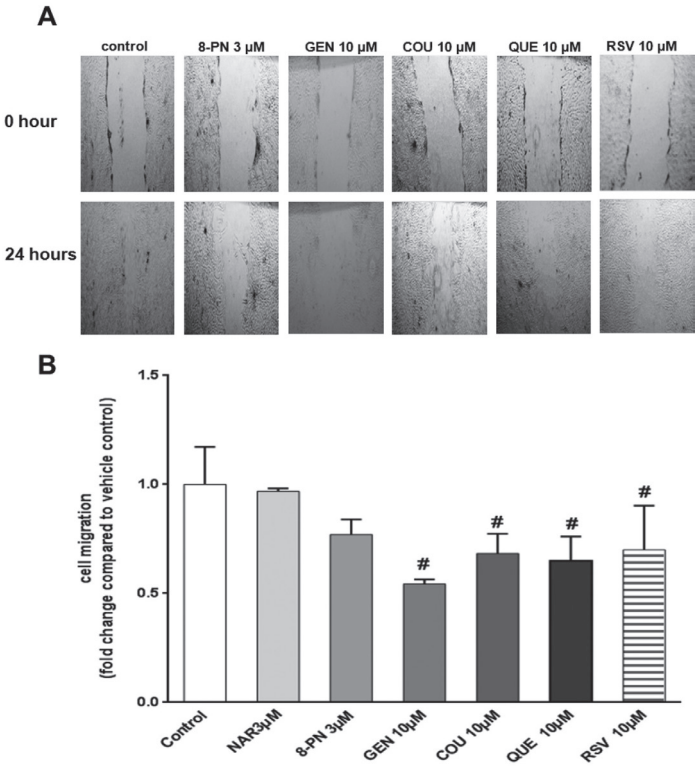

Fig. 5 Effects of phytoestrogens on KGN cell migration. (A) The examples of pictures of wound-healing assay at time O hour and after 24 hour- exposure of KGN cells to different phytoestrogens. Magnification 4X. (B) Graphical presentation of wound-healing assay. Data are shown as fold-change compared with vehicle-control treated cells. Bars represent mean+SD of three independent experiments that were performed in triplicate (N=3). * or # significantly different from relevant solvent vehicle control Ethanol (0.1%) or DMSO (0.1%) -treated cells, respectively. P < 0.05.

to the proliferative effects of phytoestrogens, routine BrdU labeling of proliferating cells was employed and KGN cells were analyzed after a 24-hour treatment with the tested phytoestrogens. No changes in cell proliferation of KGN cells were detected upon exposure to the tested phytoestrogens (data not shown). Moreover, cell cycle analysis by flow cytometry did not reveal any significant changes in distribution in phase S, G1 and G2 of KGN cells (supplementary data Fig.S.4). These results clearly indicate that phytoestrogens did not affect KGN cell proliferation nor cell cycle distribution after 24 hours.

3.5 Marginal effects of phytoestrogens on mRNA levels of genes implicated with tumor growth and progression

To investigate potential molecular mechanisms behind the inhibition of migration of KGN cells, expression of several genes that are important in tumor progression was determined. Only GEN (10 μM) statistically significantly up-regulated *VEGF* gene expression up to 1.5-fold compared with vehicle-treated control cells (supplementary data Fig.S5A). 8-PN (3 μM) caused a statistically significant 1.9-fold induction of *SIRT1* gene expression (supplementary data Fig.S5A). None of the tested phytoestrogens statistically significantly affected *MMP9* and *CADHERIN E* mRNA levels (supplementary data Fig.S5B).

4. Discussion

Phytoestrogens are plant-derived estrogen-like compounds that are increasingly used for their suggested health promoting properties, even by healthy, young women. However, scientific concerns exist regarding potential adverse effects on female reproduction. Here, we show that some phytoestrogens can modulate ovarian-specific aromatase expression and activity. Also, some phytoestrogens affected migration of human KGN granulosa-like tumor cells.

This study is the first to describe the presence and activation of the ovary-specific promoters II and I.3 in KGN cells. Within the ovary, aromatase expression is mediated primarily by gonadotropin receptors and the cyclic-AMP dependent signaling pathway, which finally contributes to an interaction and activation of the cyclic AMP response element binding protein (CREB) and steroidogenic factor 1 (SF-1) with proximal promoter II and I.3 [53]. Interestingly, it is well-known that the activation of promoters I.3 and II is a critical step for abnormal expression of aromatase levels and local estrogen biosynthesis in tumor-bearing breast tissues [54]. Both promoters I.3 and II are located closely to each other (215 base pair distance length) and are uniformly up-regulated by PGE2 via a cAMP-PKA-dependent pathway [55]. It has been well documented that in ovarian granulosa cells, CYP19A1 expression is regulated primarily by promoter II [56; 57]. We showed that PGE2 (0.1 μM) significantly up-regulated mRNA of both *I.3* and *II* promoter-driven *CYP19A1* and increased aromatase activity up to 350% when compared to vehicle-treated control cells. Similarly to PGE2, QUE (10 μM) induced *CYP19A1* expression via *I.3* and *II* promoters in KGN cells and increased *CYP19A1* activity up to 3 fold after a 24-hour exposure. Similarly, QUE (10 and 30 μM) was found to increase *I.3* and *II* -specific aromatase transcripts to approximately 2.6- and 2-fold after 24 hours exposure in human adrenal H295R cells [31]. However, in a primary culture of human granulosa-luteal cells, QUE (10μM) reduced *CYP19A1* mRNA expression in a concentration-dependent manner after an exposure period of 48 hours [28]. The same inhibitory effect of QUE upon aromatase activity was observed in human placental microsomes [58]. Interestingly, in the present study, QUE appeared to exert dual effects on aromatase at non-cytotoxic concentrations, where it stimulated aromatase activity up to 10 μM and inhibited its activity at 30 μM. This type of non-monotonic aromatase activity curve after exposure to QUE was also observed in human adrenal H295R cells [31]. In the study of Sanderson et al. (2004), the concentration of 30 μM QUE increased aromatase activity to approximately 4-fold, where after there was a sharp decline in aromatase activity. However, in that study the decline in aromatase activity was concomitant with an increase in cytotoxicity of QUE at 100 μM [31]. QUE did not cause a significant effect on estrogen production in primary cultures of human granulosa-luteal cells [59]. Also differential effects of GEN on aromatase regulation have been reported in gonadal cells. In our study, aromatase activity and protein expression remained unaltered in the presence of 10 μM of GEN. Edmunds et al. (2005) and Myllymaki et al. (2005) reported an increase of aromatase activity in human endometrial stromal cells and immature rat ovarian follicles after exposure to GEN [60; 61]. In contrast, Rice et al. (2006) demonstrated that the *CYP19A1* mRNA transcripts and aromatase activity were

reduced in human granulosa-luteal cells after exposure to 10 and 50 μM GEN [28]. In the present study, GEN (10 μM) significantly up-regulated *CYP19A1* expression, which was mostly *PI.3*-driven. Ye et al. (2009) showed a concentration–dependent induction of mRNA expression of both promoters *I.3* and *II CYP19A1* transcripts in human HepG2 cells after exposure to 10 μM GEN [62]. In human adrenal H295R cells, PII and to a lesser extent the *I.3* promoter-driven *CYP19A1* transcripts were increased by GEN at10 μM [31].

Some dietary flavanones have previously been shown to possess strong aromatase-inhibitory effects [29], nonetheless, their effects on the transcriptional regulation of *CYP19A1* and its promoter regions at mRNA level, remain unknown. In our study, 8-PN (3 μM) but not NAR (3 μM) increased mRNA levels of both *PII* and *I.3*-driven *CYP19A1* in KGN cells up to 1.8- and 2-fold, respectively. Consistently, 8-PN up-regulated *CYP19A1* mRNA transcripts, although *CYP19A1* activity was concentration-dependently inhibited (IC50 value of 8 nM). Yet, no change at protein level of *CYP19A1* was detected after a 24-hour exposure to 8-PN. This implies that 8-PN acts as a catalytic inhibitor of aromatase activity and that the 8-prenyl group is involved in inhibition of aromatase activity. It is interesting to note that the IC_{50} values for inhibition of aromatase activity by 8-PN and letrozole were in the same range. Similarly, the strong inhibitory effects of 8-PN (IC_{50} value of 100 nM) on aromatase activity was previously demonstrated by Duursen et al. (2013) in human adrenal H295R cells [63]. Further studies will need to be performed to show the nature of the aromatase inhibitory actions of 8-PN. Interestingly, our study demonstrated, that in contrast to 8-PN (3 μM), NAR (3 μM) stimulated *CYP19A1* activity but not at mRNA level. Also COU (10 μM) and RSV (10 μM) did not affect *CYP19A1* mRNA levels. COU has previously been shown to be a weak competitive inhibitor of aromatase enzyme activity in human preadipocytes [24]. In addition, the previous studies showed that RSV (50μM) inhibited the transactivation of aromatase promoters *I.3* and *II* in SK-BR-3 cells and it inhibited (IC_{50} value of 25μM) aromatase activity in MCF-7 cells [64]. Further research on phytoestrogen QUE, NAR, COU- induced aromatase activity is needed to confirm a direct involvement of CREB1 on *CYP191A* modulation. The seemingly contradictory results regarding modulation of aromatase activity upon exposure to phytoestrogens could be due the use of different *in vitro* models. As extensively described above, human aromatase is regulated in a highly tissue-specific manner. This implies that cells from different origins might respond differently to phytoestrogens. Our studies were performed using the human KGN cell line, which has previously been shown to be an excellent and applicable *in vitro* model to study effects on human granulosa cell functioning. Further studies should be performed to confirm the effects on human granulosa cells and could include primary human granulosa cells, however, be believe this is beyond the scope of our study. Also, different experimental set-up i.e. medium types, solvent used and exposure times, can contribute greatly to the observed effects. The enzymes of P450 family are highly stable proteins with half-life of 24–42 hours [65]. In contrast, the half-life of mRNA has been reported to range from 10 to 30 hours depending on tissue investigated [66; 67]. The lack of correlation between mRNA and protein levels of CYP19A1 after 24 hours in our study most likely reflect the different half-lives of mRNA and the aromatase protein.

Moreover, study of Shozu et al. (2001) provided evidence of other non-genomic modes of post-transcriptional regulation of aromatase via modulation of mitogen-activated protein kinase (MAPK) pathways [68]. Bearing in mind that phytoestrogens were shown to modulate several kinase signaling pathways including MAPK and/or Akt/protein kinase B (PKB) [69; 70], direct regulation of aromatase activity by phytoestrogens without detectable changes at mRNA or protein levels may occur. Further research is need to elucidate the modulation of aromatase activity via these mechanisms by plant-derived compounds. In the present study, we also demonstrated that phytoestrogens can induce Steroidogenic acute regulatory protein (*StAR*) mRNA levels in ganulosa KGN cells. *StAR* plays a crucial role in regulation of steroidogenesis by transporting steroid from cytosol into the mitochondria [16; 17]. All tested phytoestrogens, except NAR and GEN, statistically significantly increased *StAR* mRNA levels up to 2-fold. To date, only several studies reported phytoestrogen-mediated effects on *StAR* gene and protein expression in granulosa cells. Chen et al. (2007) reported that RSV and GEN inhibited mouse *StAR* mRNA, whereas QUE induced Star mRNA levels in murine MA-10 Leydig cells [71]. 8-PN has been shown to exert age-dependent effects on steroidogenesis by up-regulating mRNA levels of Star in progenitor and immature but not adult types of rat Leydig cells [72]. Similarly to our results, *StAR* mRNA levels were significantly up-regulated by 100 µM RSV in rat granulosa cells after 24 hours [32].

Many phytoestrogens, including RSV, GEN and QUE, have been shown to bind to both ERα and/or ERβ and induce the transcription of estrogen-responsive target genes [73; 74; 75]. Yet, despite of the structural similarity with E2, relative binding affinities of phytoestrogens for the ERα are at least 1000-10000 times lower compared to E2 and generally show stronger binding affinity for the ERβ [74]. An exception here is 8-PN. It is well-known, that NAR possess a higher relative estrogenic potency (REP) toward ERβ than ERα [76; 77]. Prenylation at the 8-position of NAR increases estrogenicity [76] and 8-PN has been described to possess a higher relative estrogenic potency (REP) in activation of ERα (10-2) than ERβ (3,9X103) [77]. Because of the known interaction of phytoestrogens with ERs and the observed effects on aromatase, we investigated whether phytoestrogens can alter aromatase expression via ER-mediated pathways. We investigated whether the known antiestrogen ICI 182,780 possess modulatory effects upon aromatase. ICI 182,780 has been shown to disrupt the nucleocytoplasmic shuttling of ERα that results in nuclear exclusion of the ER and the increase of its turnover and protein degradation [78]. The present study shows that exposure to ICI 182,780 resulted in a concentration-dependent inhibition of aromatase activity in KGN cells. Our results are in agreement with others, who also observed a concentration-dependent decrease of aromatase activity after ICI 182,780 treatment in MCF-7 cells, human fibroblasts and trophoblast cells [79; 80; 81]. The inhibitory mechanism of ICI 182, 780 upon aromatase remains unknown. However, it was demonstrated that inhibition of aromatase activity by ICI 182,780 is not via interaction with the ER, since the aromatase activity was also suppressed in ER-negative cells after exposure to ICI 182,780 [79]. This concurs with our findings that E2 alone did not affect aromatase activity in KGN cells. In our study, co-exposure of KGN cells to ICI 182,780 (0.1 µM) together with COU (10 µM), QUE (10 µM) statistically significantly

decreased aromatase activity by 46% and 73%, respectively, when compared to COU and QUE alone. Interestingly, NAR (3 μM)-mediated stimulation of aromatase activity was not attenuated by ICI 182,780 co-treatment, suggesting that the effects of NAR on aromatase is not mediated by ERα signaling pathways. It would be toxicologically relevant to further explore the mechanism behind the role of ERs in granulosa cells and modulation of aromatase activity and gene expression by phytoestrogens. KGN cells have been shown to express ERα, ERβ but also estrogen-related receptor GPER1 [82]. Recent research has highlighted the cross-talk of both genomic and non-genomic activities of ERs. Here, we have only performed studies with a selective ERα inhibitor, but future studies could include the roles of ERβ and estrogen-related receptor GPER1 as well. However, this was beyond the scope of our study.

Inhibition of E2 biosynthesis by selective blockade of the aromatase enzyme has become an established method of hormonal treatment in estrogen-dependent malignant conditions of ER-positive breast cancers. Granulosa cell tumors (GCT) are hormonally sensitive and characterize with excretion of high levels of aromatase activity and frequently secrete estrogens [83]. Treatment of women with GCT has been positively correlated with the responses to hormonal manipulations with aromatase inhibitors [84]. The interest in the potential benefits of diets high in phytoestrogens has increased, especially with regard to cancer chemoprevention. Yet, despite intense investigation, many studies give inconclusive answers whether phytoestrogens are suitable as cancer chemopreventive agents. In this study, we show that the tested phytoestrogens can affect migration of human KGN granulosa-like tumor cells as assessed by a wound healing assay. Similarly, another study described that RSV (IC_{50} of 18.8 μM) and QUE (IC_{50} of 37.5 μM) inhibited the migration of vascular endothelial cells in a concentration-dependent manner [85]. It is well known that neovascularization is a crucial factor in tumor growth, invasion, and metastasis [86; 87]. In line with this, QUE was shown to decrease VEGF secretion by myeloblastic leukemia cells NB4 [88] and possess inhibitory effects on proliferation, migration and tube formation of endothelial cells *in vitro* [85]. Also GEN was shown to potently inhibit VEGF production and suppress ovarian cancer cell metastasis *in vitro* [89]. Yet, in our study, only 10 μM GEN caused an up-regulation of *VEGF* mRNA expression. Data from previous studies demonstrated that GEN can also reduce expression of *SIRT1*. In the present study, *SIRT1* gene expression was only significantly up-regulated by 8-PN. Also, gene expression of *CADHERIN E* and *MMP9* were not affected in KGN cells exposed to tested phytoestrogens. These data show that the tested phytoestrogens reduced KGN cell migration, which is in line with the proposed cancer preventive actions of phytoestrogens. However, found no molecular pathways that could explain the inhibition of migration of KGN cells in this study and cell cycle status and proliferation were unaltered in our experimental set-up.

Upon normal dietary intake of isoflavones, the predominant group of phytoestrogens in Western countries, typically reach blood levels that are in the nanomolar range [90]. However, with vegan and vegetarian diets that are rich in isoflavones, plasma isoflavone concentrations are reported to easily reach micromolar concentrations, depending on the phytoestrogen nature and the food source [91].

Several studies have described high peak plasma levels of phytoestrogen in humans

upon dietary intake of phytoestrogens. For example, mean peak plasma levels of QUE were 0.30 and 0.74 µM after consumption of apples (325 µM) or onions (225 µM of QUE), respectively [92]. Similarly, 6 hours after ingestion of 60 g of baked soybean powder containing 112 µmol GEN, a mean peak plasma level of 2.44 ± 0.65 µmol GEN/L was detected [93]. With regard to RSV, total plasma levels of RSV were found to reach 1.33 ± 0.3 µmol/L and 1.72 ± 0.1 µmol/L after white and red wine intake, respectively [94]. Also, peak plasma concentrations of NAR of 6.0 ± 5.4 µmol/L were found in humans upon an ingestion of grapefruit juice (8 ml/kg body weight) [95]. Beside dietary intake, many phytoestrogens are also readily available as dietary supplements that can contain daily dosages up to 1200 mg of QUE, GEN or 8-PN for example [63; 96], which is several orders of magnitude higher than the dietary intake described above. It has been demonstrated that the bioavailability of soy isoflavones is higher after ingestion of soy-based supplements than after soy-rich food [97]. Several studies described peak plasma levels upon a high single oral dose of phytoestrogens. For example, a kinetic study with healthy women showed 8-PN peak plasma concentrations up to 220 nM after a single oral dose of 750 mg 8-PN [98]. Here, we described that 8-PN inhibited aromatase activity with an IC_{50} value of 8 nM, suggesting that our effect concentration is 1-2 orders of magnitude lower than reported human plasma levels for 8-PN. Still, a key obstacle in human risk assessment of phytoestrogen-containing supplements lies in the lack of human relevant pharmacokinetic data. Individual differences in gut microflora, (renal) clearance, genetic polymorphisms in metabolizing enzymes, differences in supplementation dose and type of phytoestrogen source all contribute to disparate plasma levels of phytoestrogens that have been reported in humans. Furthermore, the lack of the correlation of plasma levels with tissue concentrations e.g. in the ovary hampers proper human risk assessment of phytoestrogens. In the present study, the final concentrations of tested phytoestrogens were in the micromolar range, which is in the same order of magnitude as generally reported plasma levels upon intake of these phytoestrogens with dietary supplements. However, supplements are typically taken on a daily basis, which leads to prolonged high systemic exposure to these bioactive compounds. The complexity of phytoestrogen actions is increased by the fact that these compounds are frequently present as mixtures of several dietary components that can affect multiple signaling pathways or the same pathways resulting in additive, synergistic or opposing effects. Based on the effect concentrations from our study and the reported human plasma levels, we conclude that it is not unlikely that adverse effects on ovarian function can occur after intake of high doses of phytoestrogens via dietary supplements. Yet, for better human risk assessment of these compounds, understanding the pharmacokinetics and the combined effects of phytoestrogens on steroidogenic processes is crucial and needs to be addressed further.

5. Conclusion

Our study shows that some phytoestrogens can modulate promoter II and I.3-driven aromatase in KGN granulosa-like tumor cells, which could lead to altered ovarian estrogen production. Most strikingly were the effects of 8-PN that displayed even a more potent inhibition of aromatase activity than the therapeutic compound letrozole, while simultaneously it induced *CYP19* mRNA levels. We also showed that most phytoestrogens reduced KGN cell migration. Our study indicates that the use of dietary supplements with high contents of phytoestrogens may interfere with normal female ovarian function and that caution is in place when taking these supplements.

Acknowledgements

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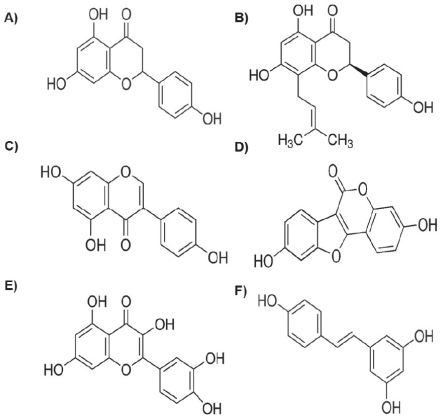
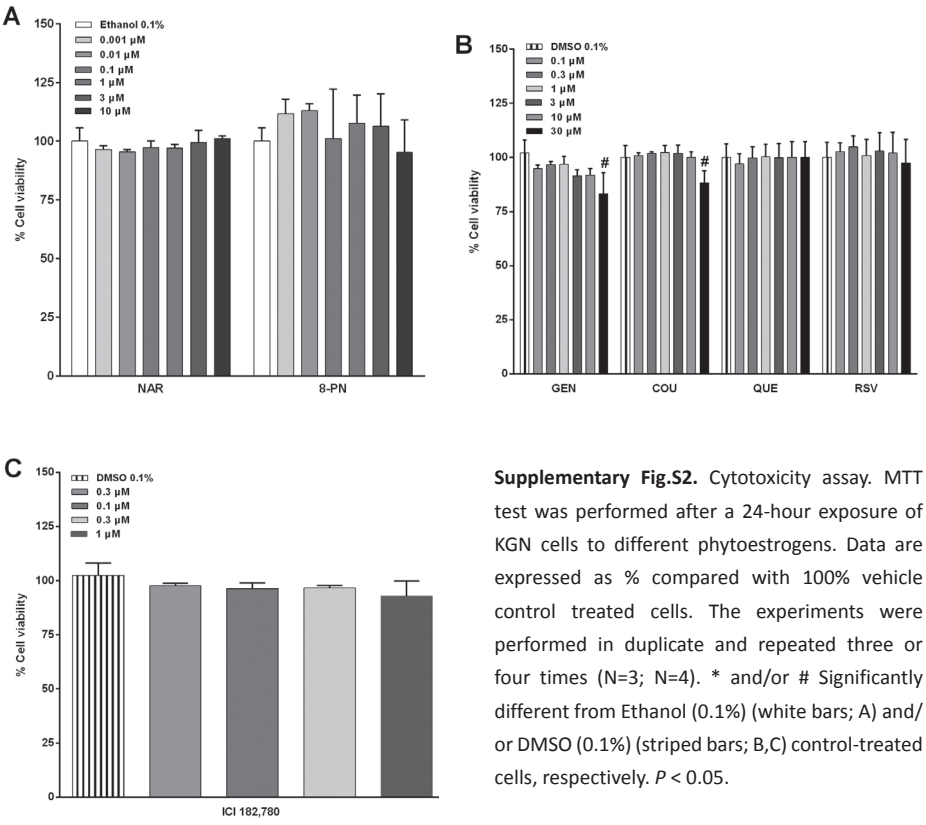
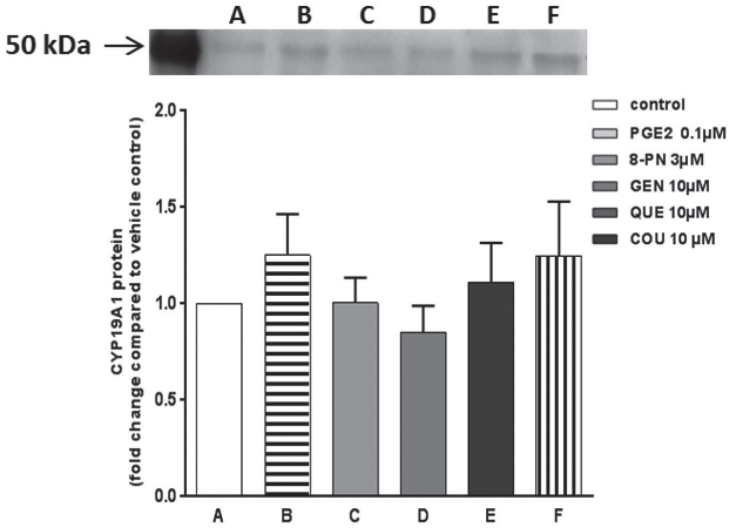
Supplementary data



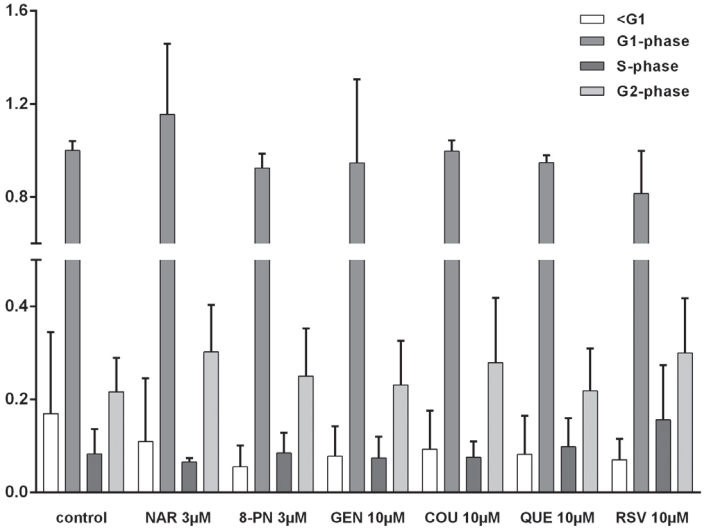
Supplementary Fig.S1. Chemical structures of tested phytoestrogens: (A) naringenin (NAR); (B) 8-prenylnaringenin (8-PN); (C) genistein (GEN); (D) coumestrol (COU); (E) quercetin (QUE); (F) resveratrol (RSV).



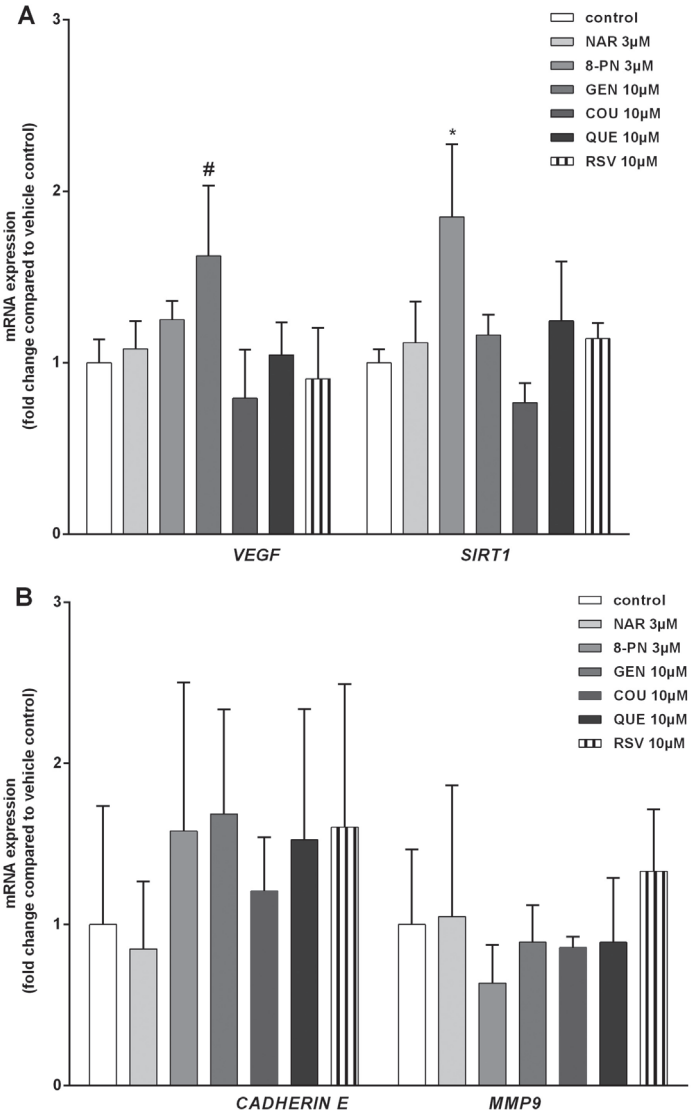
Supplementary Fig.S2. Cytotoxicity assay. MTT test was performed after a 24-hour exposure of KGN cells to different phytoestrogens. Data are expressed as % compared with 100% vehicle control treated cells. The experiments were performed in duplicate and repeated three or four times (N=3; N=4). * and/or # Significantly different from Ethanol (0.1%) (white bars; A) and/or DMSO (0.1%) (striped bars; B,C) control-treated cells, respectively. $P < 0.05$.



Supplementary Fig.S3. Western Blot analysis. 24-hour exposure of KGN cells to vehicle control (DMSO 0.1%, A); PGE2 (0.1 µM, B); 8-PN (3 µM, C); GEN (10 µM, D); QUE (10 µM, E); COU (10 µM, F). Data are expressed as fold-change compared with the average band intensity in vehicle-control treated cells. Bars represent mean+SD of two independent experiments (N=2). *P* <0.05.



Supplementary Fig.S4. Cell cycle analysis of KGN cells exposed to different phytoestrogens after 24 hours. Bars represent mean+SD of three independent experiments that were performed three or four times (N=3; N=4). *P* < 0.05.



Supplementary Fig.S5. Gene expression in KGN cells after a 24-hour exposure to diverse phytoestrogens. (A) *VEGF* and *SIRT1* mRNA levels. (B) *CADHERIN E* and *MMP9* mRNA levels. Data are expressed as fold-change compared with expression in vehicle-control treated cells. Bars represent mean+SD of three independent experiments that were performed in triplicate (N=3). * or # significantly different from relevant solvent vehicle control Ethanol (0.1%) or DMSO (0.1%) -treated cells, respectively. $P < 0.05$.

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CHAPTER 5

Naringenin (NAR) and 8-prenylnaringenin (8-PN) reduce the developmental competence of porcine oocytes *in vitro*.

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Abstract

Flavanones such as naringenin (NAR) and 8-prenylnaringenin (8-PN) are increasingly used as dietary supplements despite scientific concern regarding adverse effects on female reproduction upon excessive intake. In the present study, NAR and 8-PN (0.3-1 µM) significantly affected porcine oocyte maturation *in vitro* by decreasing cumulus expansion. In addition, NAR and 8-PN decreased percentages of meiotic spindle formation, oocyte cleavage and blastocyst formation. The effects of NAR and 8-PN were different from estradiol (3.12 µM)-induced effects. Still, the flavanone-induced effects were observed at concentrations that can be found in human plasma upon supplement intake and that resemble physiological estrogen equivalence levels in follicular fluids. Considering that abnormal oocyte maturation can cause subfertility, our study warrants that precautions are in place and excessive intake of NAR and 8-PN e.g. via dietary supplements should be avoided by women.

1. Introduction

Poor oocyte quality contributes to female subfertility and can be a key obstacle in successful *in vitro* fertilization (IVF). During fetal oogenesis, mammalian oocytes become arrested at the diplotene stage of meiotic prophase I termed the germinal vesicle (GV) stage [1]. After the onset of puberty, resumption of meiosis is induced by pre-ovulatory gonadotropin surges, and involves nuclear and cytoplasmic events leading to generation of the mature oocytes at metaphase II (MII) stage [2]. Steroid hormones, including estradiol (E2), are locally produced by granulosa cells and secreted into the follicular fluid. Steroid hormones have been implicated in maintaining oocytes at meiotic arrest [3; 4; 5] and thus controlling a possible *acquisition* of *oocyte competence* for maturation [6; 7]. Spindles that are formed during meiosis are crucial for normal chromosome alignment and separation of chromosomes [8]. Spindles are highly dynamic organelles and a disruption in tubulin synthesis and polymerization can lead to aneuploidy or maturation arrest [9; 10]. Exposure to high concentrations of E2 has been associated with aberrant meiotic spindle formation in bovine developing oocytes *in vitro* [11]. In addition, the E2 metabolite 2-Methoxyestradiol (2-ME) has been shown to bind tubulin and alter its polymerization thereby affecting spindle integrity [1]. This suggests that high levels of estrogens can be detrimental to oocyte developmental competence. Over the last decade, phytoestrogens have gained considerable attention due to their potential beneficial effects on human and animal health [12; 13; 14]. Phytoestrogens represent biologically active phenolic compounds of plant origin and are abundantly available in the diet. The structural similarity of phytoestrogens to E2 allows phytoestrogen modulation of both genomic and non-genomic estrogen receptor (ER)-*signaling pathways* [15]. Generally, phytoestrogens exert weak estrogenic activity with relative binding affinities to ERs typically ranging between 10^{-3} to 10^{-4} times the binding affinity of E2. Also, phytoestrogens usually display a higher affinity to ERβ than to ERα, in contrast with E2. Besides ER activation, these compounds may also function as anti-estrogens by blocking the binding of E2 to ERs or affecting E2 synthesis and metabolism [16; 17; 18]. Among the phytoestrogens, flavonoids are the most potent inhibitors of the estrogen-producing enzyme aromatase (CYP19) and as such were shown to interfere with E2 synthesis and release [19; 20]. Some flavonoids can also exert inhibitory effects on estrone sulfatase [21] and cytochrome P-450 isoenzymes [22]. The flavanone naringenin (NAR) is the main metabolite of naringin, which is found abundantly in citrus fruit, tomatoes, cherries, oregano, beans and cocoa, and has been shown to exert weak estrogenic activity. Prenylation at the C-8 position has been shown to significantly enhance the estrogenic properties of NAR. This is the case with the hop-derived 8-prenylnaringenin (8-PN), which is reported to be the most potent phytoestrogen known so far [23; 24]. Flavanones are present predominantly in glycosidic forms in fruits and vegetables or other plant products. Upon intake, flavanones are converted to their conjugated metabolites and transferred to the liver, where they are metabolized by phase-II enzymes before reaching the blood circulation. Intestinal absorption and gut microflora play a crucial part in determination of the bioavailability of these compounds. Moreover, distribution and accumulation of flavanones in other tissues might occur [25]. Also it has been shown that prenylation can attenuate rapid

absorption of flavonoids into the circulation by inhibiting cellular efflux [25]. Despite the many uncertainties about bioavailability of these types of compounds, several studies describe systemic levels of these compounds upon oral intake. One study describing plasma kinetics of NAR found maximum plasma levels of NAR of 6.0 ± 5.4 µM upon ingestion of grapefruit juice (8 ml/kg body weight), which corresponded to an average intake of 199 mg NAR [26]. On the other hand, the average dietary intake of 8-PN is relatively low and occurs mainly via beer. Hop flowers contain 8-PN and as a result concentrations up to 100 µg/L of 8-PN can be found in beer [27]. However, a number of hop-based supplements for example for breast enlargement containing up to 1000 mg 8-PN have recently been marketed, which potentially increases 8-PN intake of many woman at childbearing age [28]. A kinetic study with healthy women showed 8-PN peak plasma concentrations up to 220 nM after a single oral dose of 750 mg 8-PN [29].

Diets rich in fruit and vegetables have been suggested to have protective effects against chronic diseases including cancer [30]. Naringin consumption was shown to be beneficial in humans by lowering the plasma total cholesterol and LDL-cholesterol concentration in hypercholesterolemic subjects [31]. Animal studies have indicated potential advantages for 8-PN as hormone replacement therapy to improve osteoporotic bone quality [32] and alleviation of hot flashes [33]. Due to their suggested health promoting properties phytoestrogens are increasingly used as dietary supplements. This is of great toxicological concern, not only because there are still huge gaps in understanding the pharmacokinetics of phytoestrogens, but also because *several studies describe the adverse effects of phytoestrogens on female reproduction in experimental animals and humans. For example, studies have reported* early occurrence of menarche in girls fed soy products in early infancy compared with girls fed breast milk or other types of formula [34], precocious puberty in girls [35], altered estrous cyclicity in rats [36] or menstrual bleeding and increase rate of dysmenorrhea in young women [37].

The objective of the *present* paper was to study potential effects of NAR and 8-PN (for chemical structure, see supplementary data Fig.S1) on oocyte maturation and the intrinsic capacity to produce embryos after parthenogenic activation. Moreover, changes in mRNA expression of *ERα* and *ERβ* as well as steroidogenic enzymes patterns (*STAR/CYP11A1/CYP19 and HSD3B1*) were evaluated in steroid hormone producing cumulus-oocyte complexes (COCs). The pig was chosen as experimental model due to its physiological similarity to humans [38; 39]. In addition, the availability of porcine ovaries from local slaughterhouses makes the pig an attractive model to use *for reproductive toxicology testing*.

2. Material and methods

2.1. Chemicals and reagents

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA), unless indicated otherwise. Stock solutions of E2 (31.2 mM), NAR (30 mM) and 8-PN (30 mM) were prepared in ethanol.

2.2. Culture media

The culture media used were as described previously [40]. Briefly, Tyrode's lactate-HEPES-PVA medium (TL-HEPES-PVA, pH to 7.4) was used for isolation of COCs and contained 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.25 mM Na-pyruvate, 0.40 mM $NaH_2PO4.H_2O$, 10mM HEPES, 1.0 g/l of PVP, 10mM Na-lactate (syrup), 0.5 mM $MgCl_2.6H_2O$ and 2 mM $CaCl_2.2H_2O$. COCs were cultured for 42 h in *in vitro* maturation (IVM) medium, that consisted of M199 (Gibco BRL, Paisley, United Kingdom) supplemented with 2.2 mg/ml $NaHCO_3$, 0.1% (w/v) polyvinylpirolidone, 200 µM cysteamine, and 1% of follicular fluid from cyclic sows. FSH was added to the culture medium to the final concentration 0.05 IU/ml for the first 21 hours, since this hormone was shown to enhance cumulus cell expansion during the first half of the entire culture period [41]. The *in vitro* fertilization (IVF) medium containing 1 mM caffeine and 0.1% BSA (Fraction V, fatty acid free) was prepared and further kept for 18-24 hours at 38.5°C, 5% CO_2 and 7% O_2 in air before oocyte activation. For electric stimulation of oocytes, activation buffer containing 1.14 mM mannitol, and 1.15 mM $CaCl_2$, 1.16 mM MgSO4 and 1.17 mM HEPES was used. Embryos were cultured in NCSU-23 medium with 0.4% BSA. IVM and IVC media were equilibrated at 38.5°C in 5% CO_2 and 7% O_2 for at least 2 hours before culture.

2.3. Collection and culture of cumulus-oocyte complexes

Isolation and culture of cumulus-oocyte complexes (COCs) was performed as described previously [41]. Briefly, ovaries were collected from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory in a thermos flask within 2 hours. The ovaries were rinsed with running tap water and kept at 30°C in *Phosphate buffered saline* (PBS). COCs were aspirated from medium-sized (3 to 6 mm) follicles. The follicular contents were allowed to sediment and then COCs were washed in TL-HEPES-PVA. Only oocytes with compact cumulus cell content were transferred to a four-well culture dish (Nunc, Roskilde, Denmark) in groups of 30-89 COCs (See Supplementary Table.S1). The COCs were cultured in IVM medium supplemented with 0.05 IU/ml of recombinant human FSH (hFSH) (Organon, Oss, The Netherlands) in the first 21 hours of the culture. NAR and 8-PN were added to a final concentration of 0.1, 0.3, 1, and 3 µM (0.01% v/v ethanol) and were present for the whole time of maturation (42 hours). Control COCs were exposed to 0.01% Ethanol (vehicle, v/v). Similarly to the exposure with flavanones, the final concentration of E2 (3.12 µM) contained 0.01% Ethanol (v/v).

2.4. Assessment of cumulus morphology

At time 0 and 21 hours of culture, COCs were examined at 15 and 30× magnification, digitally photographed and the surface areas of individual COCs were measured using Photoshop 5.5 software (Adobe Systems, San Jose, CA, USA). The areas of the cultured COCs were expressed as mean with standard error of the mean (SEM).

2.5. Quantitative reverse transcription-PCR

After *in vitro* maturation, cumulus cells were removed from oocytes by repeated pipetting. The denuded oocytes were removed and total RNA was extracted from

cumulus cells using the RNeasy Micro Kit (Qiagen, Valencia CA, USA). Purity and concentration of the RNA samples were determined spectrophotometrically at an absorbance wavelength of 260/280nm and 230/260 nm. Complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis Kit, according to the manufacturer's instructions (Biorad, Veenendaal, the Netherlands). Obtained cDNA was diluted 10 times and stored at -20 °C until further analysis. The quantitative reverse transcription-PCR (qRT-PCR) was performed using CFX Manager (Biorad, Veenendaal, the Netherlands). The PCR was initiated by heating at 95°C for 3 min, then followed by 40 cycles with denaturation at 95°C for 15 s and annealing/ extension at 60 or 63°C for 45 s. After each run, a melt curve was performed to ensure purity of the amplicon. A negative control sample (non-RT) was included in each run. Primers for determining gene expression of *CYP19, STAR, CYP11A1, ER α, ER β, HSD3B1* have been described previously [39]. Primer sequences for pig *PTX3*: CTGCCAGCAGGTTGTGAAACA (forward) and GACCCAAATGCAGGCACTGA (reverse) were designed using Primer-BLAST of National Center for Biotechnology Information (NCBI) and then checked with BLAST (nucleotide non-redundant database) to confirm specificity. Gene expression levels were calculated using ΔΔCT method using normalization to the expression of two housekeeping genes: *GAPDH* and *PGK1*.

2.6. Nuclear maturation and microtubule organization assessment

Denuded oocytes were permeabilized for 1 hour at 30°C in a microtubule stabilizing solution, fixed with 4% buffered formaldehyde (PF; Electron Microscopy Sciences, Hatfield, PA) for a minimum of 24 hours. Next, the oocytes were stained for microtubules with a goat monoclonal anti-mouse-α tubulin antibody, dilution factor 1:100 (DAKO, Glostrup, Denmark) followed by anti-mouse IgG-Alexa 488 conjugate secondary antibody, dilution factor 1:100 (Molecular Probes). Staining for actin was performed using rhodamine-conjugated phalloidin (Molecular Probes, USA). The nuclear status of the oocytes was determined by 2.5% 4,6-diamino-2-phenyl-indole (DAPI) (Molecular Probes, Leiden, Netherlands) staining. Oocytes were mounted on microscope slides and nuclei of oocytes were examined under a confocal laser scanning-microscope (Leica TCS SPE-II). Classification of oocytes was conducted as previously described [40]. Oocytes possessing clumped or strongly condensed chromatin which formed an irregular network of individual bivalents (pro-metaphase) or a metaphase plate without a polar body were classified as being at the MI stage. Oocytes showing condensed chromatin in a metaphase plate with microtubule spindle formation together with a separate polar body were classified as being at the MII stage. Spindle distance length was analyzed by determining the length between two poles and calculated using 3D viewer of Image J 1.47c software (National Institutes of Health, USA). For each spindle length, the average of 3 measurements was used. Only oocytes with spindles with two poles were analyzed.

2.7. Lipid droplet staining of oocytes

Following *in vitro* maturation, denuded oocytes were fixed with 4% buffered formaldehyde and stored at 4°C for at least 24 hours. Lipid droplets staining was performed as described previously [42; 43]. Briefly, oocytes were washed twice in

PBS with 0.3% (w/v) polyvinylpyrrolidone (PVP), permeabilized for 30 min in PBS with 0.1% (w/v) saponin (PBS-S; Riedel-de Haën, Seelze, Germany) and 0.1 M glycin (Merck) and washed once with PBS-S. The specific neutral lipid stain BODIPY 493/503 (Molecular Probes) in PBS (20 µg/ml, 1 hour) was used to stain lipid droplets. Oocytes were washed three times in PBS with 3 mg/ml of PVP and nuclei were visualized by 2.5% DAPI staining. Oocytes were then mounted using a 0.12-mm eight-well Secure-Seal Spacer (Molecular Probes) on a glass slide (Superfrost Plus; Menzel, Braunschweig, Germany), covered in Vectashield (Vector Laboratories, Burlingame, CA) and sealed with a microscope slide (Superfrost Plus).

2.8. Activation of oocytes by electric impulse and embryo development

After IVM, oocytes were denuded, washed in mTBM medium and incubated in activation buffer for 10 min. Electro-activation was performed using an Electrofusion instrument CF-150B (BLS, Budapest, Hungary) at room temperature (RT). Oocytes were placed in a chamber filled with activation buffer, in a single row between the electrodes and activated by two consecutive pulses of 50 mV during 80 µsec. The activated oocytes were transferred to 60 µL drops containing 14-59 oocytes per drop (See Supplementary Table.S2) of 5 µg of cytochalasin-D-containing IVC medium under mineral oil for 3 hours in 5% CO_2 and 7% O_2 at 38.5°C. Next, activated oocytes were washed three times in IVC medium and placed in IVC medium (500 µL per well, 4 well plates). The oocytes were subsequently transferred to NCSU-23 with 0.4% BSA and cultured for 6 days.

2.9. Data analysis

Data were obtained from at least three independent experiments. Specific number of oocytes and biological replicates are given in the figure legends and in supplemental Table.S1 and S2. Prior to statistical analysis, the F-test and/or Bartlett's test were used to assess the equal variations of samples in each performed experiment. Analysis of statistical significance in differences of the means between vehicle-treated control and flavanone-treated oocytes was performed using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) multiple-comparison test. Statistical analysis of difference of the means between vehicle-treated control and estradiol-treated oocytes was determined by Student's t-test. Data were analyzed using Prism 6.0 (Graph Pad Software, San Diego, CA, USA). For each experiment, differences were considered statistically significant at P values <0.05.

3. Results

3.1 NAR and 8-PN impair cumulus expansion

To determine the effects of NAR and 8-PN on cumulus expansion, COCs were exposed to final concentrations of 0.1, 0.3, 1 and 3 µM NAR or 8-PN during IVM. At the start of culture (time 0), the projected surface areas of individual COCs was measured. After 21 hours of the COCs culture with flavanones, the surface of each

single exposed COC was re-measured. The ratio of the COC surfaces in time (21/0 hours) was calculated for each exposure and the relative cumulus expansion was presented as a fold change over ethanol (0.01% v/v) control (Fig.1E). NAR caused a statistically significant decrease in COCs expansion, causing a 26% ($p<0.05$), 32%; $p<0.004$) and 31% ($p<0.007$) reduction at 0.3, 1 and 3 µM, respectively, when compared to vehicle control-treated COCs. Exposure to 8-PN (1 and 3 µM) also reduced cumulus expansion to 24% ; $p<0.04$) comparing to vehicle-treated control cumulus (Fig.1E). Light microscopic images demonstrate that COCs cultured with the highest concentration (3 µM) of NAR and 8-PN contained more condensed and shrunken cumulus investment (Fig.1C,D). To determine whether the observed reduction of cumulus in flavanone-exposed COCs was due to the presumed estrogen-like properties of the compounds, E2 (3.12 µM) was used as positive control. However, this high concentration of E2 did not statistically significantly affect cumulus cell expansion (Fig.1B,E).

3.1.1 Neither PENTRAXIN 3 mRNA expression, nor mRNA of BAX/BCL2 ratio in COCs are affected by flavanones.

To further elucidate how the flavanones may impair cumulus expansion, *PENTRAXIN 3 (PTX3)* mRNA levels were evaluated in NAR- and 8-PN-exposed porcine cumulus cells from COCs after 42 hours of maturation. *PTX3* is involved in the assembly of extracellular matrix of the cumulus cell layer [44]. The levels of *PTX3* mRNA in cumulus cells were not affected when oocytes were exposed to E2 (3.12 µM). Similarly, there were no significant changes in *PTX3* mRNA expression in cumulus cells cultured with NAR (0.1 – 0.3 µM) (supplementary data, Fig.S2). In contrast, treatment with 1 µM 8-PN statistically significantly up-regulated *PTX3* gene expression. To evaluate whether the observed depletion of cumulus cells was due to the activation of apoptotic pathways, the ratio of *BAX/BCL2* mRNA were determined in cumulus cells as well as *CASPASE 3* (*CASP3*) mRNA levels. There were no statistically significant changes detected in *BAX/BCL2* mRNA ratio (supplementary data Fig.S3) nor in *CASP3* gene expression (data not shown).

3.2 Marginal changes in steroidogenic gene expression triggered by flavanones.

To evaluate possible modulatory effects of the tested flavanones on the impairment of steroidogenesis, mRNA levels of key steroidogenic enzymes were investigated upon exposure of COCs to NAR and 8-PN for 42 hours. Exposure to E2 (3.12 µM) statistically significantly decreased *CYP19* mRNA expression ($p<0.0001$), while both flavanones increased *CYP19* mRNA expression (Fig.2). Exposure to 8-PN (0.3 µM) resulted in a statistically significant induction of *CYP19* mRNA of approximately 2-fold ($p<0.05$). The highest concentrations of 8-PN increased *CYP19* mRNA, yet not statistically significantly. Similarly, the increase of *CYP19* mRNA expression was not statistically significantly different from vehicle-treated control cells at all tested concentrations of NAR, nonetheless a similar trend to 8-PN exposed COCs was observed (Fig.2). There was a trend toward a decrease of *StAR, CYP11A1* and *HSD3B1* mRNA levels, but no statistically significant changes in mRNA expression of these

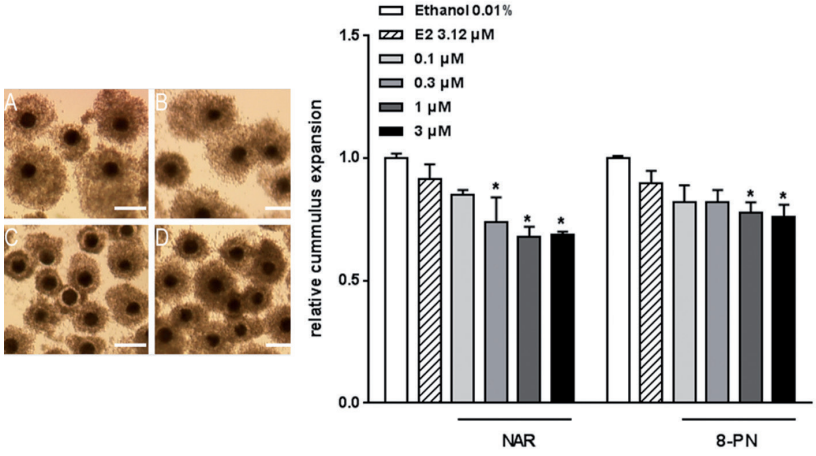

Fig.1 Morphology of porcine cumulus oocyte complexes (COCs) exposed to 0.01% Ethanol (A), 3.12 µM E2 (B), 3 µM NAR (C) or 3 µM 8-PN (D) at 21 hours. Scale bars represent 200 µm. Dose–response effects of NAR (left) and 8-PN (right) on porcine cumulus expansion (E). Data are presented as fold changes compared with vehicle-treated control (0.01% Ethanol) COCs. Bars represent mean+SEM of three independent experiments. * Significantly ($P < 0.05$) different from control cells. Number of oocytes used per treatment in three independent experiments is given in the supplementary Table S1.

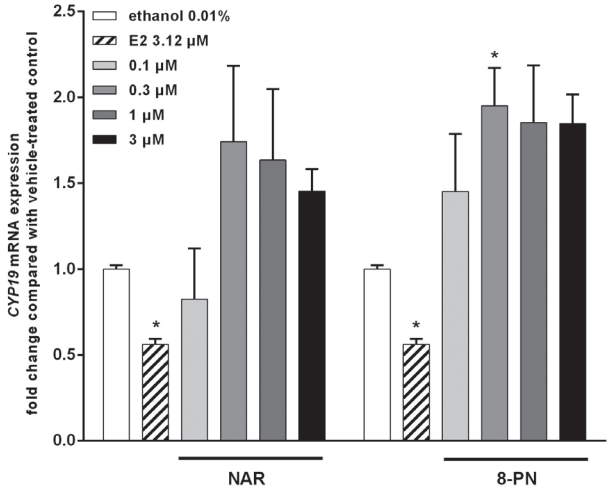

Fig.2 Relative expression of *CYP19* mRNA in cumulus cells exposed to estradiol (E2, 3.12 µM) or various concentrations of naringenin (NAR) or 8-prenylnaringenin (8-PN). Data are expressed as fold changes over ethanol (0.01% v/v) vehicle-control. Bars represent mean+SEM of four independent experiments. * Significantly different (P< 0.05) from vehicle-control. Numbers of oocytes used for cumulus cell isolation per treatment in four independent experiments are given in the supplementary Table S1.

genes were observed in NAR- and 8-PN–exposed cumulus cells (data not shown). Because the cellular ERα/β ratio is suggested to determine the ultimate effect of phytoestrogens on cell proliferation [45], this ratio was determined in cumulus cells. Interestingly, E2 (3.12 μM) decreased the ER α/β mRNA ratio to 0.65±0.01 in cumulus cells. Both 8-PN and NAR did not have an effect on ERα/β ratio (Fig.S4).

3.3 Flavanones affect oocyte nuclear maturation by impairing spindle formation

Nuclear maturation refers to the progression of the oocyte nucleus from the germinal vesicle breakdown (GVBD) until the second meiotic arrest at MII. The establishment of bipolar spindles is an essential event of meiosis in oocytes. The majority (90%) of vehicle-treated control oocytes reached the MII stage and showed normal spindle formation after 42 hours of culture (Fig.3A; Fig.4A,B). Exposure to E2 (3.12 μM) significantly affected spindle formation (Fig.3F; Fig.4A,B). The distance between the poles of formed spindles in E2-treated oocytes was statistically significantly reduced ($p<0.0005$) to 67% compared with 100% vehicle-treated control oocytes (Fig.4C,D). This effect on spindle formation might be due to the reduction of tubulin content (Fig.3F, white arrow) or an improper alignment of chromosomes (Fig.3F; blue arrow). Also NAR caused a concentration-dependent decrease in spindle formation in exposed oocytes up to 52% at 1 μM concentration of NAR ($p<0.0001$) (Fig.3B-E; supplementary data Table.S1). The effects of 8-PN on spindle formation appear to be biphasic. Oocytes exposed to lower concentrations of 8-PN (0.1 and 0.3 μM) contained more disorganized spindles with scattered chromosomes and irregular spindles (Fig.3H; blue arrow) or even a complete lack of spindle formation, even though the microtubules were clearly present (Fig.3H; white arrow). In oocytes exposed to 8-PN (0.1μM), the statistically significant 35% reduction ($p=0.001$) of the distance between two spindle poles compared to the spindle distance of control oocytes, was measured. Particularly in 8-PN (0.3 μM)-treated oocytes disorganized tubulin in the nucleus and lack of bipolar spindle formation was apparent (Fig.3H; Fig.4D). However, the effects on spindle formation and spindle distance in oocytes exposed to higher concentrations of 8-PN were less severe (1 and 3 μM, respectively) (Fig.4D).

3.4 NAR but not 8-PN affects lipid droplets in porcine oocytes

Porcine oocytes contain glycogen granules and lipid inclusions used as energy source during oocyte growth and maturation and embryo development. In the present study, lipid droplet distribution was evaluated in porcine oocytes treated with NAR or 8-PN (final concentrations: 0.1, 0.3, 1 and 3 μM) during IVM. In 90% of vehicle-treated control oocytes, lipid droplets were uniformly distributed (Fig.5A) and some clumping lipid droplet mass was observed (Fig.5K). Treatment with E2 (3.12 μM) resulted in more clustered lipid structures and fused-like lipid content was apparent (Fig.5F,L). Similarly to E2, NAR concentration-dependently induced clustering of lipid droplets (Fig.5B-E,M). The effects of 8-PN exposure on lipid droplet aggregation and fusion were not as clear as for NAR-treated oocytes (Fig.5G-J). Nonetheless, fused-like structures of lipid droplets were observed in 0.3 and 1 μM 8-PN-treated oocytes (Fig.5N).

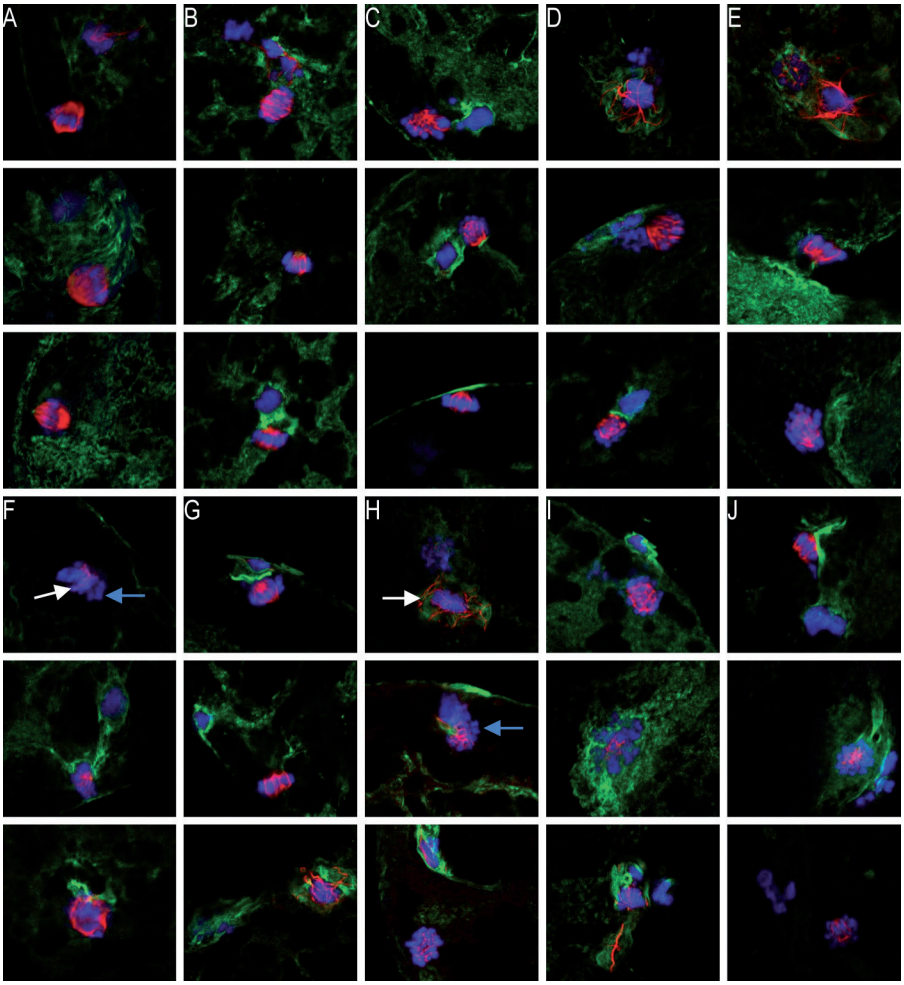

Fig.3 Confocal laser scanning photomicrographs of organization of microtubules/microfilaments in porcine oocytes treated with 0.01% Ethanol (A), 0.1 µM NAR (B), 0.3 µM NAR (C), 1 µM NAR (D), 3 µM NAR (E), 3.12 µM E2 (F), 0.1 µM 8-PN (G), 0.3 µM 8-PN (H), 1 µM 8-PN (I), 3 µM 8-PN (J). Three different oocytes are presented for each exposure. Green, microfilaments; red, microtubules; blue, chromatin. White arrows show the reduction of tubulin content (F) and lack of spindles (H). Blue arrows show the scattered chromosomes (F;H). Magnification X 400.

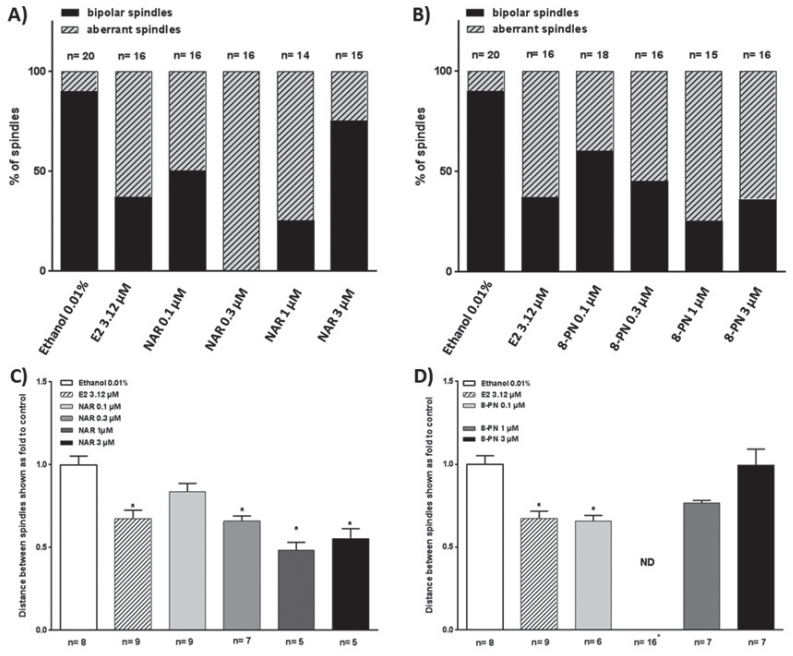

Fig.4 Graphical presentation of % of normal (bipolar) and aberrant spindles calculated for each exposure (for the details, see supplementary Table S1). Exposure to NAR (A) and 8-PN (B). Effects of flavanones: NAR (C) and 8-PN (D) on spindle distance in porcine oocytes at MII. ND, no spindles were formed and no spindle distance could be measured. * Significantly (P< 0.05) different from vehicle-treated control. Total number (n) of analyzed oocytes. # No statistical analysis could be performed due to the lack of spindle formation.

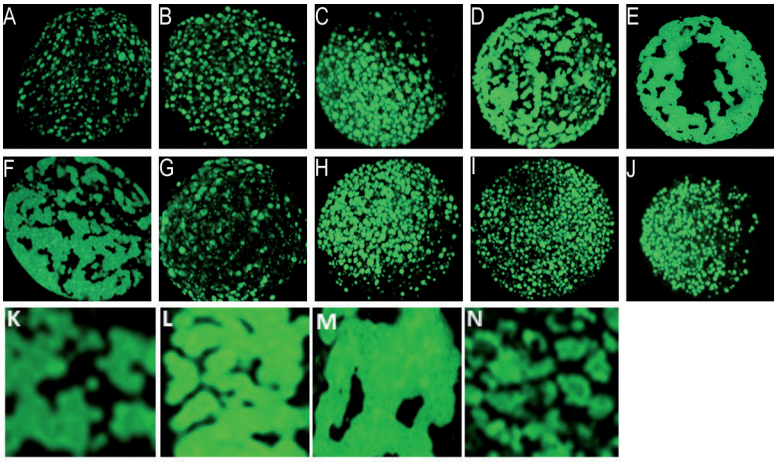

Fig.5 Confocal laser scanning photomicrographs of organization of lipid droplets in porcine oocytes treated with 0.01% Ethanol (A), 0.1µM NAR (B); 0.3 µM NAR (C); 1 µM NAR (D); 3 µM NAR (E); 3.12 µM E2 (F); 0.1µM 8-PN (G); 0.3 µM (H); 1 µM 8-PN (I); 3 µM 8-PN (J). Green, lipid droplets; blue, chromatin. Magnification is X 400. The organization of lipid droplet clusters in porcine oocytes. Exposure to 0.01% Ethanol (K); 3.12 µM E2 (L); 3 µM NAR (M); 0.3 µM 8-PN (N). The number of analyzed oocytes per treatment was 5-7 per each independent experiment (N=3).

3.5 Impairment of oocyte cleavage results in decrease of blastocyst formation after flavanone exposure

A decrease in cumulus cell expansion has been associated with decreased fertilizability and developmental capacity [46]. In order to avoid possible sperm-related effects, parthenogenetic activation of oocytes was chosen to study effects of NAR and 8-PN on embryo development. No reduction in developmental capacity was observed after exposure to E2 (3.12 µM) (Fig.6A,B). Cleavage of oocytes was slightly decreased when oocytes were matured in the presence of 8-PN (Fig.6A,B). More pronounced effects were observed in NAR-exposed oocytes (Fig.6A,B). NAR statistically significantly decreased oocyte cleavage up to 50% at 3 µM ($p < 0.005$) when compared to vehicle-treated control oocytes. A similar pattern in decrease of blastocyst formation was seen with NAR and 8-PN treated oocytes. No changes in blastocyst size were found for all treatments (data not shown).

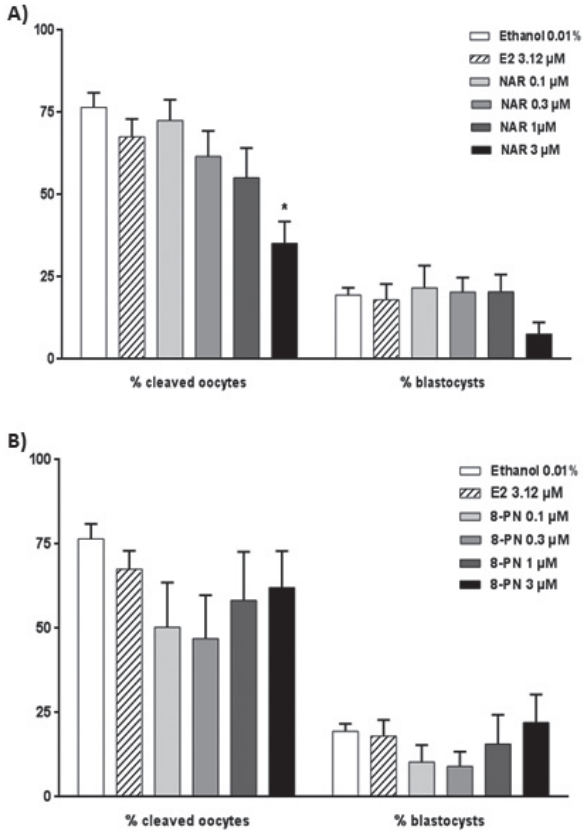

Fig.6 The effects of flavanones: NAR (A) and 8-PN (B) on embryo development. Graphs represent % of cleaved oocytes % blastocysts of whole activated oocytes. Bars represent mean+SEM of four independent experiment. * Significantly ($P < 0.05$) different from vehicle-control. The number of oocytes per treatment used in each experiment is given in the supplementary Table S2.

4. Discussion

In this study, we demonstrate adverse effects of NAR and 8-PN on *in vitro* oocyte maturation and competence of oocytes to develop into blastocysts using porcine immature COCs. These findings imply that excessive exposure to certain phytooestrogens can reduce fertility.

During pre-ovulation, a marked expansion of cumulus takes place in COCs, which is prerequisite for proper ovulation. In this study, cumulus cell expansion was inhibited and the morphology of flavanone-treated cumulus layer appeared to be more compact. During mucification, cumulus cells secrete an extensive extracellular matrix amount consisting of hyaluronic acid (HA), extracellular matrix proteins and proteoglycans [44]. It has previously been demonstrated that flavonoids are able to inhibit the hyaluronidase activity in bovine testis and mouse sperm [47; 48]. In addition, NAR was shown to prevent degradation of HA through the inhibition of hyaluronidase [49]. It is therefore unlikely that the decrease in cumulus cell expansion after exposure to NAR and 8-PN in this study was due to the degradation of HA.

PTX3 functions downstream of the Growth differentiation factor 9 (GDF-9) signal transduction cascade and is involved in cumulus matrix stability [50]. PTX3 is produced by cumulus cells and expressed in the extracellular matrix, where it plays a key role in retaining HA molecules in the intercellular spaces [44]. Our qRT-PCR data did not reveal significant changes of *PTX3* mRNA levels after treatment with NAR. An up-regulation of *PTX3* mRNA expression was observed in 1µM 8-PN treated COCs but the biological significance of this observation remains unknown.

NAR can trigger the mitochondrial-mediated apoptosis pathway as demonstrated by an increased ratio of (pro-apoptotic) BAX/(anti-apoptotic) BCL2, subsequent release of cytochrome C and sequential activation of CASP3 [51]. We however did not detect any signs of apoptosis that could explain the reduced cumulus expansion in our study. The morphology of lipid droplets in oocytes was altered after exposure to NAR in a concentration-dependent manner. At concentrations ≥ 0.3 µM of this flavanone, lipid droplets became more clustered and appeared to be fused. Typically, lipid droplets contain a mixture of different types of lipids surrounded by a single layer of phospholipids of varying composition, but the amount of droplets and lipid content varies between species. Especially porcine oocytes are rich in lipid droplets compared to humans. During meiotic maturation, fertilization and early embryonic development, a rearrangement of lipid droplets occurs in porcine oocytes [52]. Flavanones possess amphipathic properties and are able to partition into lipid bilayers and to alter the membrane structure [53]. NAR has also been shown to affect cholesterol metabolism in rats and in HepG2 cells thereby lowering lipid levels and exerting insulin-like properties [54; 55]. Although it must be noted that the total amount of lipid content was not determined in our study, any imbalance in lipid droplet composition may potentially affect oocyte competence and developmental potential. Indeed, clustering of cytoplasmic organelles including lipid droplets has previously been observed in abnormal oocytes of rhesus monkey and rabbit [56; 57] and abnormally developing mouse embryos [58].

Within the follicle, E2 production continuously changes during the ovarian cycle *it has been demonstrated that phytoestrogens can affect steroid production in vitro* [59; 60]. Therefore, we studied the effects of NAR and 8-PN on the steroidogenic gene expression profile in cumulus cells. Steroidogenesis initiates at the inner mitochondrial membrane, where the cytochrome P450 cholesterol side chain cleavage enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone. The ability of cholesterol to move into mitochondria to be available for CYP11A1 is under control of steroidogenic acute regulatory protein (StAR), which determines the efficiency of steroid production. Our study revealed that the mRNA expression of *StAR* and *CYP11A1* remained fairly constant after the treatment with both flavanones. The levels of *HSD3B1* mRNA in NAR and 8-PN treated COCs were also similar when compared with those in vehicle-treated control oocytes (data not shown). A crucial enzyme in steroidogenesis is aromatase (CYP19), which is responsible for the conversion of androgens into estrogens. Various research groups, including ours, have described aromatase induction or inhibition by phytoestrogens in *in vitro* cell systems [61; 62]. The flavanones are considered to be potent inhibitors of aromatase activity [59]. *Strong inhibition of aromatase activity by* 8-PN (IC50 value of 0.1 μM) has been reported in human adrenal H295R cells, even though CYP19 gene expression was increased [59]. Similarly, NAR has been shown to act as competitive aromatase inhibitor in various *in vitro* systems [63; 64]. NAR inhibited aromatase activity with IC50 values ranging from 0.7 to 4 μM in MCF-7 breast cancer cells [65]. Nonetheless, the effects of NAR and 8-PN on *CYP19* transcription remain unknown. In the present study, increased *CYP19* mRNA levels have been found for all tested concentrations of both flavanones. It has been documented that porcine aromatase exists as three distinct isoforms that arise from multiple genes and complex splicing [66]. In contrast, human aromatase is encoded by a single gene and displays tissue-specific expression that arises from alternate promoter usage [67]. In light of the above, the relevance for human risk assessment of the effects of NAR and 8-PN on CYP19 gene expression described here remains to be investigated. Moreover, further studies should focus on phytoestrogen effects on aromatase activity in granulosa cells, since appropriate physiological concentrations of E2 are essential for acquisition and maintenance of cumulus cell competence to undergo expansion [42]. Yet, how E2 signaling interacts with other factors to affect follicular growth and cumulus expansion remains unknown. An increase in cumulus expansion has been demonstrated in murine COCs exposed to E2 (0.1 μM) *in vitro* [47]. In the present study, a high level of E2 (3.12 μM) did not affect cumulus expansion in porcine COCs. This high E2 concentration was chosen based on previous studies from our group showing that this concentration inhibited COC maturation and caused spindle abnormalities after 30-40 hours [11]. After a 21-hour exposure, this high concentration of E2 did not significantly affect porcine cumulus expansion. In contrast, NAR and 8-PN significantly reduced porcine cumulus expansion at concentrations of 0.3 up to 3 μM and 1 to 3 μM, respectively. It is known that NAR and 8-PN display relatively weak estrogen receptor agonist actions. The relative estrogenic potency (REP) for 8-PN was shown to be 100 times less than E2 as estimated using yeast that stably expressed human ERα or β [68]. Importantly, 8-PN is relatively more potent on ERα than ERβ, as its REP for ERβ

activation was estimated to be 3 orders of magnitude lower than for ERα [68]. 8-PN was approximately 4 orders of magnitude less potent than E2 in an ERα-reporter gene assay and ER-dependent proliferation in a MCF-7/H295R co-culture model [59]. NAR exhibits very weak estrogenic activity in the rat uterus and was estimated to be more than $2.5Xl0^{4}$ times less potent than E2 [69]. The *in vitro* estrogenic potency of NAR was 10^{-3} to 10^{-4} relative to that of E2 in a reporter-gene assay [70]. Contrary to 8-PN, the REP of NAR for ERβ ($5.2X10^{-5}$) was marginally higher than for ERα ($<10^{-5}$) in estrogen receptor assays [24; 68]. Taken together, this means that the estrogenic equivalence of NAR and especially 8-PN at which inhibition of cumulus expansion was observed in this study lies in the nM range. This is well within the physiological range of estradiol levels that can be found in porcine follicular fluids, which have been detected at 200 ng/ml (≈ 0.73 μM) [71]. Moreover, the finding that effects were observed by low estrogenic equivalent compounds 8-PN and NAR but not high concentrations of E2 concurs with the non-monotonic dose-response curves that can typically be observed in hormonal responses.

A high E2 concentration has previously been shown to cause spindle abnormalities in porcine oocytes *in vitro* [11]. Also in bovine oocytes, *in vitro* exposure to E2 caused nuclear aberration manifesting in delays in the formation of a typical bipolar spindle at meiosis I and induced severe spindle aberrations at metaphase II [72; 73]. Most of the E2-mediated effects on spindle formation were found to be caused by 2-methoxyestradiol (2-ME), a metabolite of E2 [1]. The formation of multipolar spindles is characteristic for 2-ME-exposed oocytes and indicates disruption of microtubule kinetics and their organization [1]. In this study, we also detected atypical, three polar spindles in approximately 10% of the E2-exposed oocytes, but not in NAR and 8-PN-treated oocytes (data not shown). Thus, it is unlikely, that the observed changes in spindle formation by NAR and 8-PN were 2-ME-mediated as a result of increased E2 metabolism. Moreover, both NAR and 8-PN are inhibitors rather than inducers of CYP1A1/1B1 CYP450 enzymes involved in the phase I of E2 metabolism [74; 75]. Although the morphology of the meiotic spindles in flavanone-exposed oocytes did not resemble spindles of oocytes exposed to E2, NAR and 8-PN did affect spindle formation. Exposure of oocytes to NAR resulted in concentration-dependent decrease in percentages of normal spindle formation. In contrast, 8-PN appeared to have a biphasic effect on spindle formation. At 0.3 μM 8-PN, the largest adverse effect was observed and tubulin remained mainly scattered in or around dispersed chromosomes. When oocytes were exposed to higher concentrations of 8-PN (≥1μM), tubulin reorganization was less affected, although chromosomal dispersion of metaphase spindle structure was still observed. Even though the effects of NAR and 8-PN appear not to be 2-ME-mediated, an ER-mediated effect on spindle formation cannot be ruled out considering the biphasic responses and low estrogenic potencies of NAR and 8-PN as described above.

For human risk assessment purposes, it is important to note that the concentrations of NAR and 8-PN in this study are within the range of human plasma levels that can be found upon ingestion. It is clear from pharmacokinetic studies that both NAR and 8-PN are readily absorbed, reaching maximal plasma levels already after 1-5 hours. Also, human plasma levels in the low micromolar range have been reported for NAR

and 8-PN. Yet, it should be noted that existing knowledge gaps on pharmacokinetics clearly hamper proper human risk assessment of phytoestrogens. For example, large interindividual differences in plasma levels and area under the curve (AUC) of phytochemicals exist due to variations in gut microflora populations, polymorphisms in metabolizing enzymes and variations in (renal) clearance and enterohepatic cycling [76; 77]. Furthermore, it is not known how plasma phytoestrogen levels correlate with follicular fluid levels. Substantial phase II metabolism of NAR and 8-PN occurs upon absorption, which can substantially affect the target dose levels of NAR and 8-PN. Human studies show NAR-glucuronides in urine but not in plasma, suggesting renal glucuronidation of NAR in humans as opposed to rats that display high plasma NAR-glucuronide levels [78; 79; 80]. Also, abovementioned human NAR and 8-PN plasma levels are derived from single dose studies, while our *in vitro* studies more resemble continuous exposure scenarios. Despite these unceratinties, the pharmacokinetic parameters still indicate that it is reasonable to assume that upon repeated high intake, e.g. via dietary supplements, plasma levels in the low micromolar range can be achieved similarly to our *in vitro* test situation.

5. Conclusion

Taken together, our findings demonstrate that NAR and 8-PN cause abnormal oocyte maturation by affecting the cumulus-oocyte complex and impairing the developmental competence of porcine oocytes *in vitro*. These effects appear to be a result of a disturbance in meiotic spindle formation and are most likely not an estrogen-like effect via the E2 metabolite 2-ME, but an ER-mediated effect cannot be ruled out. Moreover, exposure to NAR induced an apparent accumulation and fusion of lipid droplets in porcine oocytes. Exposure to the flavanones NAR and 8-PN decreased oocyte cleavage and blastocyst formation at estrogen equivalence levels that are in the physiological range of estradiol levels in porcine follicular fluids. Despite the proposed beneficial effects of dietary flavanones on human health, our study warrants that precautions are in place and excessive intake of NAR and 8-PN e.g. via dietary supplements should be avoided.

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Supplementary data

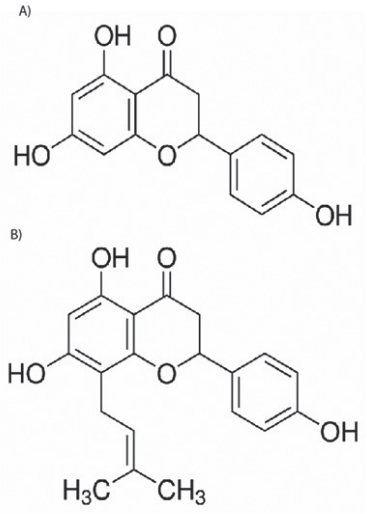

Fig.S1 Chemical structures of naringenin (NAR) (A); 8-prenylnaringenin (8-PN) (B).

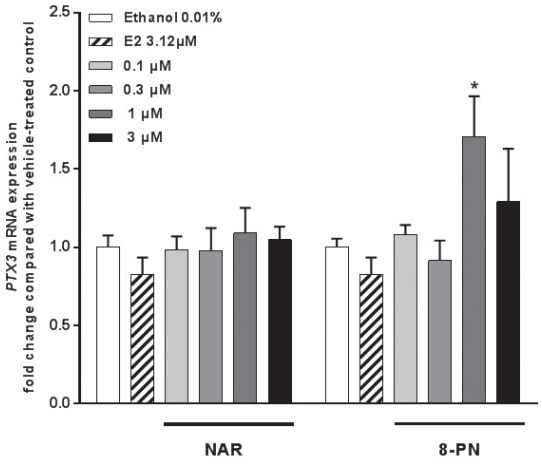

Fig.S2 *PTX3* mRNA expression in porcine cumulus cells exposed to E2 (3.12 µM; striped bar) or different concentrations of NAR and 8-PN. Data are presented as fold changes over Ethanol (0.01%) vehicle-control. Bars represent mean+SEM of four independent experiments. * Significantly (P <0.05) different from vehicle-control. The number of oocytes used for cumulus cell isolation per treatment is given in the supplementary Table S1.

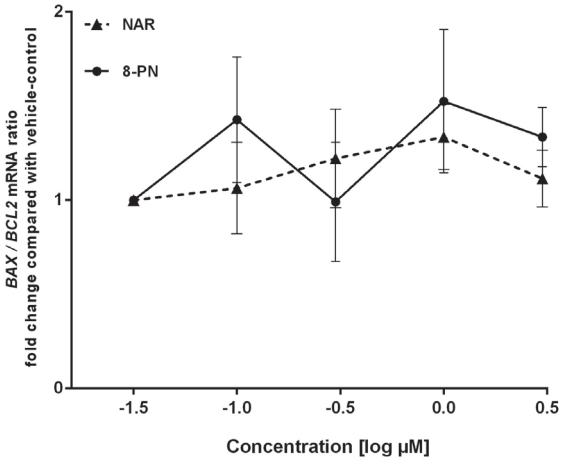

Fig.S3 The ratio of BAX/BCL2 mRNA expression in cumulus cells exposed to flavonoids. Data are expressed as fold changes over ethanol (0.01%) vehicle-control. Bars represent mean+SEM of four independent experiments. The number of oocytes used for cumulus cell isolation per treatment is given in the supplementary Table S1.

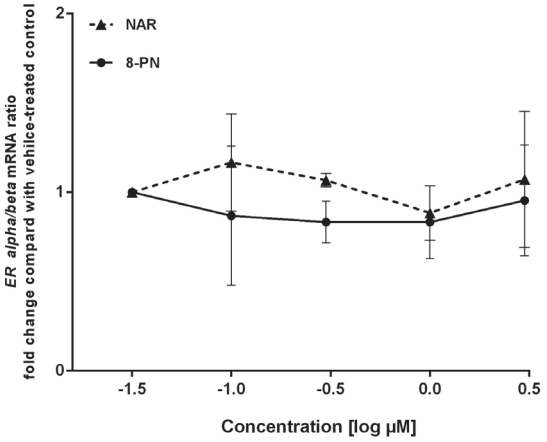

Fig.S4 *ERα/β* mRNA ratio. Data are expressed as fold changes over ethanol (0.01%) vehicle-control. Bars represent mean+SEM of four independent experiments. # significantly ($P < 0.05$) different from vehicle-control. The number of oocytes used for cumulus cell isolation per treatment is given in the supplementary Table S1.

Supplementary Table S1. (*) The number (n) of cumulus-oocyte complexes (COCs) used for cumulus expansion evaluation. (#) The number (n) of COCs used for gene expression study of cumulus cells in each independent experiment per different treatment.

Independent Experiment	Ethanol (0.01%)	E2 (3.12 µM)	NAR (0.1 µM)	NAR (0.3 µM)	NAR (1 µM)	NAR (3 µM)	8-PN (0.1 µM)	8-PN (0.3 µM)	8-PN (1 µM)	8-PN (3 µM)
Exp. I *# n =	89	50	84	70	70	82	74	53	53	71
Exp. II *# n =	64	38	61	64	71	55	49	57	55	79
Exp. III*# n =	66	61	69	65	53	67	58	69	77	69
Exp. IV # n =	47	58	57	46	53	59	52	47	52	30

Supplementary Table S2. Embryo development. The number (n) of oocytes used in each independent experiment per different treatment.

Independent Experiment	Ethanol (0.01%)	E2 (3.12 µM)	NAR (0.1 µM)	NAR (0.3 µM)	NAR (1 µM)	NAR (3 µM)	8-PN (0.1 µM)	8-PN (0.3 µM)	8-PN (1 µM)	8-PN (3 µM)
Exp. I n =	50	52	42	31	24	48	28	43	30	40
Exp. II n =	27	24	29	37	40	47	64	56	32	50
Exp. III n =	40	32	40	49	45	43	43	59	39	38
Exp. IV n =	31	42	26	21	26	14	41	30	27	43

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CHAPTER 6

Summary & Discussion

Summary

1. Introduction

The endocrine system regulates vital processes crucial for the control of growth, development and reproduction in all vertebrates. For the assessment of endocrine disrupting chemical (EDC) exposure, the HPG axis is of particular interest, as there is a strong indication that some EDCs may affect normal regulation and activity of this dynamic organ system leading to the alterations among the hypothalamic, pituitary, and gonadal components. In females, this might further result in perturbations of cyclicity and has an impact on female sexual development and functioning. The mechanisms of EDCs action involve modulation of different cellular pathways of which Estrogen Receptor (ER)- and Aryl hydrocarbon Receptor (AhR)- mediated cellular responses in the reproductive tract are highly conserved in wildlife and humans. Although, there have been numerous *in vitro* and *in vivo* testing methods developed to screen EDCs, our knowledge especially with respect to mechanistic understanding of endocrine modulation on the HPO-axis, is limited. Reproduction involves the interactions among many diverse cell and organ systems of the body. The data from human studies is limited and the safety evaluation of toxic effects of EDCs upon reproductive system is based on *in vivo* studies. *The animal* studies are clearly cost and time- consuming and from an ethical point of view are being questioned. Therefore, there is a strong need for further development of *in vitro* methods in this area.

2. Goal

This thesis has two main objectives:

1. The development of *in vitro* models for testing endocrine disruption in hypothalamus- pituitary -ovary (HPO) axis,
2. The mechanistic-based *in vitro* evaluation of endocrine disruptive effects of known EDCs such as dioxins and phytoestrogens on hypothalamus, pituitary and ovary level.

To fulfill these aims, this thesis has been subdivided into four separate studies. The main outcomes of the research are presented below.

Chapter 2 describes the applicability of the use of primary cell cultures of rat hypothalamus, pituitary and gonadal origin to investigate the adverse effects of dioxin and non-dioxin compounds in all HPO compartments. The aim of the study was to explore to which extent dioxin (TCDD), dioxin-like (PCB126) and non-dioxin-like (PCB153) compounds can affect the main components of rat HPO-axis and if the *in vitro* primary cell responses are predictive for the *in vivo* outcomes. The results of this study are in agreement with the available *in vivo* data on dioxin-like compounds effects in reproductive system. In addition, **Chapter 2** provides information about

mechanistic action of these toxic compounds on the HPO system. The results indicate that the AhR-mediated effects of dioxins can cause perturbations in all tested *in vitro* systems, with the most pronounced effects observed at ovarian level. At hypothalamus level, Kiss-1 neurons were shown to be dioxin and non-dioxin targets in the brain. At pituitary and ovary level, mainly the FSH signaling pathway was disturbed by dioxin-like compounds. The significant alterations of mRNA expression of *Cyp17* and *Cyp19A1* by dioxin-like compounds indicate a possible direct modulation of AhR- mediated action by dioxin-like compounds on ovarian steroidogenic enzymes. To fully understand the mechanistic basis of dioxin-like action, functional analysis of AhR-targeting e.g. using siRNA approach need to be performed. The siRNA AhR –targeting technique has been successfully introduced in **Chapter 3**. It is well known that the AhR is involved in many biological and toxicological responses, including neuroendocrine signaling. Currently, a suitable *in vitro* model to study potential effects of exogenous compounds at the cellular and molecular level in the hypothalamus is lacking. A hypothalamic model or cell line should ideally express AhR. This study introduces a new AhR-expressing rat hypothalamic GnV-3 cell line as a cell model for AhR-mediated neuroendocrine modulation in hypothalamus. **Chapter 3** provides several important findings which demonstrate a number of AhR-dependent pathways associated with the circadian rhythm and food intake control, but not with 5-HT receptor regulation. Also, data presented in this study reveals that GnV-3 cells follow an approximate 24-hour circadian rhythm, which exhibits a similar pattern as *Gnrh* mRNA. In conclusion, the GnV-3 cell line may be considered as an additional tool for future screening of the neuroendocrine effects of other AhR ligands at the hypothalamus level.

With respect to potential adverse effects on female reproduction, scientific concerns have been raised regarding potential reproductive adverse effects of phytoestrogens. These plant-derived estrogenic compounds are increasingly used for their suggested health promoting properties, even by healthy, young women. The research in **Chapter 4** was conducted on human tumor derived granulosa KGN cells, which were shown previously to be an excellent applicable *in vitro* model to study effects on human granulosa cell functioning. The results of this research reveal that phytoestrogens can affect various pathways in granulosa-like cells by inducing *StAR* mRNA levels and modulating ovarian-specific promoter II and I.3 aromatase expression and activity. Most of tested phytoestrogens reduced KGN cell migration, although molecular mechanisms underlying this outcome require further investigation. KGN cells proved to be a valuable cell model for evaluation of EDCs at ovary level. In **Chapter 5** the investigation on adverse effects of phytoestrogens on reproductive function in ovary is continued. **Chapter 5** introduces a useful and promising porcine oocyte *in vitro* model for reproductive toxicity testing. In **Chapter 5**, effects of the flavanones naringenin and 8-prenylnaringenin in this model are described. The findings of this study demonstrate that NAR and 8-PN cause abnormal oocyte maturation by affecting the cumulus-oocyte complex and impairing the developmental competence of porcine oocytes *in vitro*. The exposure of oocytes to NAR caused a concentration-dependent decrease in percentage of normal spindle formation. In contrast, 8-PN appeared to have a biphasic effect on spindle formation. Importantly, the exposure

to NAR and 8-PN decreased oocyte cleavage and blastocyst formation at estrogen equivalence levels that are in the physiological range of estradiol levels in porcine follicular fluids. Strikingly, concentration-dependent accumulation and fusion of lipid droplets in porcine oocytes were observed after NAR treatment. Despite some uncertainties arising from the existing knowledge gaps on pharmacokinetics of phytoestrogens, we show in **Chapter 4** and **5** that phytoestrogens may interfere with ovarian function and oocyte maturation at concentrations that can be found in plasma upon supplement intake. These results indicate that excessive intake of phytoestrogens e.g. via dietary supplements should be avoided by women.

Finally, the future perspective with respect to animal alternatives and *in vitro* battery testing aimed at reproductive toxicity were described in Part III of the discussion. The focus has been put on development of integrated intelligent testing strategies for EDCs testing. This chapter describes the possible applicability of integrated computational PBPK/PBPD model of the HPG axis for assessment of EDCs. This can be achieved by the integration of *in vivo, in vitro* (using reverse dosimetry approach) and human clinical data, to better predict complex concentration-duration-response relationships and chemical–receptor interactions occurred within this axis. The data obtained from such a model can serve as a potential tool for the improvement of the understanding of the dynamic behavior of this axis and for better extrapolation of results from *in vivo* and *in vitro* studies to humans. Also, the integrated PBPK/PBPD model could be used for earlier identification of potential or new molecular biomarkers for chemical screening of endocrine modulation at regulatory level.

Discussion

Normal reproductive function requires precise temporal and quantitative regulation of hormone secretion at all levels of the hypothalamic–pituitary–ovary (HPO) axis in the female. Thus, understanding the physiological processes covering the entire reproductive axis is crucial for the assessment of toxic effects of chemicals. The focus in this thesis was put on Estrogen Receptors (ER)- and Aryl hydrocarbon receptor (AhR) - mediated regulation of the female reproductive tract. ERs are mediating cellular responses of circulating estrogens during the female cycle and ER subtypes were shown to exert various biological effects at the tissue level. The AhR, on the other hand has been shown to play an important role in controlling the development of the reproductive axis by regulating normal function of the organs of the female reproductive system from fetal development to adulthood [1; 2]. Unfortunately, reproductive toxicology studies that assess effects on the HPO-axis require the use of many animals. Currently, only a limited number of *in vitro* models have been proposed to study interference of EDCs with either the hypothalamus, pituitary or ovary functions. Still, none of these *in vitro* systems have been thoroughly studied and validated with respect to *in vivo* relevance. Furthermore, there is currently no suitable *in vitro* model to investigate the actual interaction within compartments of the HPO-axis.

Clearly, when evaluating some reproductive and developmental toxicological endpoints such as litter size of newborns, weight of organs, cyclicity patterns and other *in vivo* data are still needed. Also, at the regulatory level, the assessment of toxicological effects is still based on *in vivo* animal studies and if possible human data, if applicable. However, for elucidation of the molecular mechanisms underlying the disruptive action of dioxins on the reproductive tract, an *in vitro* approach is certainly more favored.

This thesis has been dedicated to evaluate possible tools for testing modes of action of EDCs on neuroendocrine and steroid hormone modulation of the HPO-axis *in vitro*. In this chapter, we firstly will outline the *in vitro* models that have been used in this project (PART I). Subsequently in PART II, the effects will be described of some dioxins and phytoestrogens, which are well-known EDCs affecting AHR and ER-pathways. Both classes of compounds have been shown to exert modulatory *in vivo* effects on the reproductive tract. Finally, PART III provides information about possible future directions for other non-animal testing of environmental chemicals on the reproductive axis.

PART I. *In vitro* cell models for the HPO-axis in this thesis

1.1 Hypothalamus *in vitro*

The hypothalamus is a key control center responsible for all endocrine functions, including regulation of hormonal feedbacks within the HPO-axis. Understanding of the reproductive control mechanisms of these unique hypothalamic peptidergic neurons is critical for a proper assessment of endocrine modulation. With respect

to the reproductive axis, hypothalamic Kiss-1 and GnRH neurons are the principal hormone- releasing neurons responsible for regulation of key events of reproduction. The study of GnRH neuronal physiology within the hypothalamus *in vivo* was proven to be very difficult due to their low number, wide distribution and scattered location within the hypothalamus [3; 4].Yet, for mechanistic and toxicological purposes, a good *in vitro* model for the hypothalamus is of great value. The dissociated primary neuron cultures have been a popular research tool for decades [5; 6]. Chapter 2 describes the application of successful primary adult rat hypothalamic cell culture. A prerequisite for this *in vitro* approach was the fact that primary cell cultures can retain a differentiated function for a short period of time and can have characteristic of intact neurons *in vivo*. Despite that animals are still needed, more exposures can be performed with one single animal, thus reducing the number of animals. Therefore, the use of primary cell cultures for toxicity testing shall bring more reliable prediction of toxic effects observed *in vivo* than e.g. with immortalized cell lines [7]. However, during the isolation process loss of integrations between the neuronal networks occurs. Furthermore, the viability of primary cell cultures is much shorter than in immortalized neuronal cell lines, which can be easier maintained in culture. However, we showed that our primary culture neurons kept their functional gene characteristics and morphology for 10 days. These cultures were applied in our study with dioxins and dioxin -like compounds at the hypothalamus level (Chapter 2).
The focus of this thesis was not only limited to primary cell culture models. There are several *in vitro* hypothalamic cell models described in the literature [for review: 8]. Also, several commercial cell line models of rodent hypothalamus origin have been recently developed. As the scope of this thesis aimed to investigate AhR-mediated neuroendocrine effects in the hypothalamus *in vitro*, we studied an *in vitro* hypothalamic cell model for its potential use in testing the AhR-mediated neuroendocrine effects by dioxins (Chapter 3) [9]. Most *in vitro* studies that investigate the hypothalamus use the murine GT1-7 cell line, yet this cell line does not express the AhR [10]. Therefore, we investigated the applicability of the GnV-3 cell line in Chapter 3. This hypothalamic rat cell line expresses the AhR, which is similar to the *in vivo* situation. GnV-3 cells were generated by a modified Tet-On system to condition the expression of the immortalizing agent *v-myc* to the presence of doxycycline in the culture medium [11]. This transformation allows proliferation of GnV-3 cells in the presence of doxycycline, whereas in the absence of doxycycline, the cells exhibit many features of mature, non-dividing, differentiated GnRH neurons [11; 12]. Contrary, most available GnRH-releasing hypothalamic cell lines, such as GT1-7, were generated from developing murine GnRH neurons using the SV-40 large T antigen transformation system targeted to the *Gnrh* promoter region, and therefore may not entirely resemble the differentiated adult neurons due to loss of the post-mitotic stages [13]. The absence of the neuronal architecture complexity makes hypothalamic immortalized cell lines and cultures limited compared to the whole brain. However, both immortalized GnRH neuronal cells and isolated hypothalamic GnRH neurons may serve as valuable *in vitro* tools to define many membrane and nuclear factors important for HPO-axis regulation [14]. Both primary hypothalamic neurons and GnV-3 cells exhibit a GnRH ‘pulse generator’ that mediates reproductive

competence as reported *in vivo* [15, 16]. Both cell types also contain a set of important neurotransmitter- and endocrine receptors such as Kiss-1-R, GnRH-R, ERβ, ERα and glutamate (*Grik*$_2$), gamma-amino butyric acid (*Gabra*$_2$) and serotonin (*Ht*$_{2c}$), respectively. Finally, they contain the whole functional AhR system for AhR-mediated action of dioxins, which will be described further in PART II for primary and GnV-3 cells, respectively. However, culturing the whole hypothalamus in order to assess disrupting effects of EDCs on the Kiss-1 system, has a major limitation. Two main Kiss-1 populations of neurons can be distinguished in the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV), which have been shown to give opposite responses to E2 [17]. In our study, whole rat hypothalamus was cultured and no distinction was made between ARC and APVN neurons, which can potentially lead to over- or underestimating an effect.

1.2 Pituitary *in vitro*

Together with the hypothalamus, the pituitary exerts a considerable influence over the function of other endocrine glands, including ovaries. In the endocrine cells in the anterior pituitary, a pulsatile release of the hypothalamic hormone GnRH stimulates gonadotropin secretion, such as luteinizing and follicle stimulating hormones (LH and FSH, respectively), via a G-protein coupled receptor (GnRHR) and subsequent increase in cytosolic Ca^{2+}. There are currently only a limited number of cell lines that are suggested to be good representatives of the gonadotroph phenotypes. For instance, RC-4B cells comprise all anterior pituitary cell types including corticotropes, thyrotropes, gonadotropes, somatotropes and lactotropes except folliculostellate cells [18; 19]. Also, RC-4B cells were shown to be morphologically similar to that of differentiated anterior pituitary cells [20]. Our analysis of the expression pattern of genes crucial for HPO regulation revealed that RC-4B cells express both FSH and LH, GnRH-R, which is in agreement with immunocytochemical examination done by others [18; 21]. However, low detectable gene and protein expression of FSH and LH, as evaluated by us using qRT-PCR and ELISA, limited the applicability of these cells for further toxicological assessment in our present studies. In contrast, primary pituitary rat cultures do display these gonadotrophic characteristics. In chapter 2 we successfully used these cultures as pituitary *in vitro* model to assess the neurotoxic and reproductive toxic potential of various compounds including EDCs. Primary pituitary cultures have been extensively used and described by others as well [22; 23; 24]. At present, the contribution of primary pituitary cells on biomedical and toxicological research cannot be ignored anymore.

1.3 Ovary *in vitro*

The ovary is the most dynamic tissue system in the female body during its reproductive life span. Within the ovary, two primary steroidogenic cell types can be distinguished: theca and granulosa cells. These cells synthesize and secrete hormones (steroids and growth factors) that are essential for the inter-relationships between other organs of the HPO-axis as well as the central nervous system (CNS). The follicle is a functional unit of the ovary that consists of an oocyte surrounded by theca and granulosa cells. According to the two-compartment model, theca cells of

maturing follicles are capable of producing androgens in response to the LH surge. Subsequently, androgens are converted into estrogens including estradiol (E2) by FSH-induced aromatase in granulosa cells [25]. The use of primary ovarian granulosa and/or theca cells has been introduced long time ago. Since then, granulosa-thecal cell cultures have been used to study ovarian physiology and to assess the pharmacological and toxicity impact of chemicals at ovarian level [26; 27]. During folliculogenesis, follicles exhibit distinct patterns of gonadotrophin sensitivity and acquire an increased ability to produce androgens and convert them to estradiol (E2) in response to basal gonadotropin concentrations. Thus, to increase the sensitivity of these cellular responses to EDCs, cells from rat ovaries in diestrus (late I and II) were used (Chapter 2).

With respect to *in vitro* granulosa and/or theca models, several available cell lines have been introduced so far [28]. One very interesting cell line is the KGN granulosa-like tumor cell line of human origin (Chapter 4). KGN cells were previously shown to maintain many of the physiological features of normal human granulosa cells, including steroidogenesis [29] and secretion of estrogens [30; 31]. In Chapter 4 we demonstrated for first time that KGN cells display the ovarian-specific PII/I.3-driven aromatase expression, similarly to normal granulosa cells. Additionally, KGN cells have also been reported to express both ERs similar to the human vivo situation [32]. Moreover, several studies have demonstrated that KGN cells respond similarly to primary human granulosa cells upon stimulation with e.g. FSH [29; 33]. Therefore, we found this cell line to be the most suitable *in vitro* model to study phytoestrogen effects on human ovarian steroidogenesis.

Obviously, any effect of a compound at ovarian level can also include a direct effect on the developing oocyte. Several ways exist how chemicals can disrupt oocyte development. During meiotic progression of the oocyte, chromosomes have a bipolar spindle for their further segregation during both meiotic divisions. In case of any disruption e.g. by a chemical agent, this will likely lead to impairment in chromosome pairing or spindle formation. To study this, we have examined the porcine cumulus-oocyte culture bioassay. This model is extensively described in Chapter 5 of this thesis. The porcine cumulus-oocyte culture bioassay was chosen as experimental model due to its physiological similarity to humans. In addition, this model requires no extra sacrificing of animals since porcine ovaries used for our experiments were obtained from local slaughterhouses.

PART II. Endocrine disrupting compounds (EDCs) and the reproductive axis

The Globally Harmonized System (GHS; rev. 5) defines reproductive toxicity as “adverse effects of chemicals on sexual function and fertility in adult males and females” [UNECE, 2013, Part 3; 34]. The further discussion of this chapter will address the issue of adverse effects of dioxins and phytoestrogens at HPO-axis observed *in vivo* and their correlation to effects detected in *in vitro* assays discussed in PART I.

2.1 AhR-mediated effects by dioxins on the HPO- axis

The numerous animal and human epidemiological data on dioxins and PCBs have shown, that exposure to these compounds can cause a variety of adverse effects on sexual development and physiology of the reproductive system in experimental animal species [35; 36; 37]. Among others, dioxins were shown to deplete serum levels of estrogens, disrupt estrous and/or menstrual cyclicity resulting in decreased fertility in rats [35; 38; 39; 40] and monkeys [36; 41; 42]. However, these adverse effects of dioxins on reproductive function have mainly been shown at higher doses *in vivo*. Less consistent evidence on dioxin and PCB action on the changes of steroid hormone patterns, are obtained from human data. Effects on reproduction and early life stage development in humans remains limited, but a change in sex ratio at birth with an excess of females to males in the most TCDD-contaminated area of Seveso after an industrial incidence has been documented [43].

Without doubt, most of the effects of dioxin-like compounds are AhR-mediated. The accumulation of these congeners in the brain including hypothalamus has been well documented in rodents following administration of dioxins [44; 45; 46; 47]. For example, after a single oral dose of TCDD (10 µg/kg), the protein and mRNA levels of *Cyp1a1* were time-dependently increased in several brain regions including hypothalamus as well as in the pituitary, with the highest level observed 1 day after TCDD treatment in male Sprague–Dawley rats [45]. Similarly to the *in vivo* situation, we found changes in induction pattern of *Cyp1a1* and *Ahrr* mRNA that were concentration-dependent in GnV-3 cells exposed to TCDD (0.1-10nM) within 24 hour-time period (Chapter 3). The highest induction level of *Cyp1a1* mRNA up to 600 fold was observed after a 4-hour exposure and time-gradually decreased and reached approximately 7 fold after 24 hours [9]. Also, an increase in *Cyp1a1* and *Ahrr* mRNA levels was observed in primary cell cultures of hypothalamus, pituitary exposed to TCDD (10nM) and PCB126 (100nM) (Chapter 2). In the ovary, AhR expression has been found in the theca and granulosa compartment as well as in the oocyte [48]. The well-balanced expression and activity of AhR is essential for normal ovarian function [31]. The presence of dioxins in human ovarian follicular fluid and their well-documented activation of AhR [49], (Chapter 2) implicate the direct action of these compounds at ovarian level. Moreover, the well-studied inhibitory crosstalk between AhR and ER signaling, offers a possible direct effects of dioxins in the alteration of ovarian functions, including changes in steroidogenesis with further alteration of ovulation. Indeed, TCDD has been shown to act as an anti-estrogen *in vivo* and *in vitro* through the modulation of E2 metabolism via the activation of CYP1A1 and/or CYP1B1 [50; 51]. This anti-estrogenic characteristic of TCDD could lead to the decrease of E2-induced responses, such as decrease of uterine weight [52], cell proliferation [53], and changes in regulation of nuclear receptors [54; 55].

The *in vivo* neuroendocrine effects of dioxins have been well demonstrated at hypothalamus level [40]. As described in more detail in PART I, the hypothalamus is the site for synthesis and secretion of numerous key neuropeptides involved in regulation of the HPO-axis. Kiss-1 neurons are well-documented gatekeepers of the onset of puberty and regulator centers of GnRH secretion and represent important modulators of the HPO-axis [56]. As demonstrated by others, a disruption within the

Kiss-1 neuronal network leads to abnormal pubertal timing and alteration of menstrual cycle [57; 58; 59]. When this disturbance occurs during critical developmental windows, such as the pre-pubertal period, the occurrence of metabolic diseases has been reported [60; 61]. Up till now, only a few reports pointed at Kiss-1 neurons as a potential biological target for exposure to dioxin-like compounds [62; 63]. In chapter 2 we demonstrated that exposure of primary hypothalamic cultures to TCDD (10nM) and PCB126 (100nM) resulted in up-regulated levels of *Kiss-1* mRNA. Our results strongly implicate direct disrupting effects of dioxins on Kiss-1 neurons, which might be a potential novel biological target for dioxin exposure.

GnRH hormone is one of the most studied endocrine markers with respect to physiology of reproductive axis. The disruption of the normal functioning of the hypothalamic circuitry may lead to an interruption of GnRH synthesis. TCDD was shown to influence daily *in vivo* fluctuations of *Gnrh1* via disturbance of the hypothalamic GABA-ergic neurons [64]. GnRH neurons express only ERβ [65], which similarly to ERα, has been implicated in the cross talk between dioxin and estrogen signaling in human T47D cells [66]. Thus, those neurons might serve as potential target for some environmental endocrine disruptors. Also, our study demonstrated, that exposure to TCDD significantly affected mRNA expression of *Gnrh1* in a time- and AhR-dependent manner in GnV-3 cells. In contrast, *Gnrh1* gene expression was not affected by TCDD in rat primary cultures (Chapter 2). However, as demonstrated in Chapter 3, *Gnrh1* mRNA oscillates *in* time-dependent manner, and thus *Gnrh1* expression should be determined at multiple time-points in order to clarify whether dioxins are capable of disturbing the daily *Gnrh1*pattern in primary hypothalamic cultures. At the pituitary level, the regulation of FSH and LH is under control of specific pulsatile pattern of GnRH, that in rats in the afternoon of pro-estrous cause a significant surge of gonadotropins from the anterior part of this organ. As shown by Gao et al, (2000), exposure to environmentally relevant concentrations of TCDD induced a significant reduction of LH and FSH secretion. However, this blockage of gonadotropin surge was partially overcome after the treatment with GnRH during the preovulatory period in female rats [67]. This may imply that the changes in GnRH pattern could be a result of direct TCDD action to the central nervous system (CNS). Similarly to Kiss-1 neurons, the circadian biological clock has been shown to be one of the important modulators with respect to regulation of the basal GnRH pulse frequency, particularly in the generation of gonadotropin surges [68; 69]. Indeed, we provide in Chapter 3 for the first time the evidence of a synchronized pattern of Period circadian protein homolog 1 (*Per1*) and *Gnrh1* gene expression in GnV-3 cells is presented. *Per1* gene expression is also affected by TCDD in a time dependent manner, which is in agreement with results from anonther *in vivo* study demonstrating AhR-mediated disruption of circadian genes activity [70]. Within the neuronal network, ERα and AhR-expressing GABAergic neurons are another set of neurons, which play a crucial role in onset of puberty and regulation of GnRH neurons [71]. The presence of ERα on those neurons may implicate them as potential targets for dioxins exposure. Interestingly, the distribution pattern of AhR gene expression overlaps with that of glutamic acid decarboxylase (GAD) 67, the enzyme essential for gamma-aminobutyric acid (GABA) synthesis. Indeed, developmental exposure

to TCDD explicitly down-regulates GAD 67 expression in the rPOA/AVPV in female rats resulting in abolished sexual differentiation of this area [72, 73]. Interestingly, our study demonstrated a significant induction of *Gabra2* and *Grik2r* receptor gene expression after 4h exposure to TCDD (10nM). Other results from *in vivo* studies also indicate that TCDD impairs NMDA receptors, which are known to play an essential role in memory formation and maintenance [74; 75]. In addition, it was shown that the homeostasis of serotonin (5-HT) and its precursor tryptophan was disturbed in the brain and plasma of rats after exposure to TCDD [52; 76; 77]. Prolonged pre-exposure to TCDD (10 nM) up to 24 h accompanied with the continued elevation of Ht_{2c} mRNA expression in GnV-3 cells however did not significantly affect 5-HT secretion (9; Chapter 3). Also, it is well known that the wasting syndrome, i.e. decreased food intake and progressive weight loss, is a main hallmark of dioxin exposure observed in rats [78; 79] and in monkeys [80]. Moreover, the evidential AhR-mediated effects of TCDD on neuropeptide levels have been clearly demonstrated for instance with respect to orexigenic neuropeptide Y neurons [81; 82], which contain AhRR-positive nuclei [82] and are generally regarded as one of the main regulators of eating [83]. The *in vivo* studies showed elevation of mRNAs of NPY after a TCDD dose (15 µg/kg) in Sprague Dawley rats or TCDD (50 µg/kg) in Long-Evans rats. However, the changes in mRNA of NPY observed after TCDD treatment were transient, which possibly resulted in a compensatory reaction to ongoing body weight loss of animals observed in the latter study. This concurs with our *in vitro* data on GnV-3 cells, which demonstrated transient 2.3-fold increase of NPY mRNA levels [9; Chapter 3].

With respect to the pituitary, TCDD has been shown to alter gonadotropin secretion in rats. LH and FSH release is crucial for the regulation of sexual development and gametogenesis. So far, contrary data has been obtained from rat *in vivo* experiments, which showed either increase of FSH and LH surge [10; 39; 84] or inhibition of their release [67] after exposure to TCDD. These data support a direct action of dioxin-like compounds on the pituitary, but the exact mechanism of this effect remains elusive. Also PCB126 was shown to modulate the pituitary function by increasing LH and FSH secrection in male rats. However, a concomitant reduction of plasma gonadotropin levels was detected, which lead to conclude that this congener has a direct effect on the alteration of the secretory mechanisms at pituitary level [85]. Using a primary pituitary culture model, we demonstrated that both TCDD and PCB126, but not PCB153, significantly up-regulated the mRNA levels of *Fshbeta* but not *Lhbeta*. The presence of the AhR in the pituitary and increase of *Fshbeta* mRNA by TCDD and PCB126 suggests that the AhR may play a role in this process. These results point to FSH as one of the endocrine targets of dioxin-like compounds in females (Chapter 2). Interestingly, several studies have indicated a crosstalk between AhR and FSH pathways in the transcription of the *Fshr* gene expression, as a consequence of the presence of a AhR-responsive element in the promoter regions of the *Fshr* [86; 87;88]. In addition, a recent study, Teino et al., 2014 demonstrated that the expression of *Ahr* in mouse granulosa cells is modulated by gonadotropins during follicular development [89]. As mentioned in PART I, FSH plays an important role in the promotion of the expression of steroidogenic gene i.e. CYP17 and CYP19A1, from which the latter is involved in E2 synthesis in granulosa cells [90]. At environmentally

relevant (10 pM) concentrations of TCDD, this compound significantly inhibited FSH-induced FSH-R mRNA expression in rat granulosa cells at 24 hrs [91]. Furthermore, E2 secretion was significantly altered by TCDD (1 pM or 3.1 nM) treatment in human luteinized granulosa cells [92]. In our study with primary granulose-theca cells only TCDD (10nM) and not PCB126 nor PCB153 statistically significantly reduced mRNA levels of *Cyp17* and *Cyp19*. However, no changes in E2 secretion were detected (Chapter 2).

Taken together, our data show that exposure to dioxins and dioxin-like PCBs can cause a wide spectrum of neuro-endocrine perturbations in experimental animals. Functions of several hypothalamic neurons can directly be affected by dioxins. Moreover, dioxins can act on FSH signaling via direct actions on pituitary FSH secretion as well as by modulating ovarian responsiveness to FSH. This again strengthens the notion that (neuro)-endocrine effects should be investigated at the entire HPO-axis rather than at single compartment level. Although the transcriptional activation of P450 family members by AhR ligands is well documented, additional pathways of AhR-mediated actions by dioxins in the brain have been proposed such as elevation of intracellular Calcium [Ca^{2+}]i and induction of oxidative stress. The future studies focusing on the non-genomic action of dioxin-like compounds would help explain the complexity of dioxin effects in the human body.

3. The effects of phytoestrogens at ovarian level

It is well accepted that individual phytoestrogens can exert different cellular actions that are considered to be species- and tissue-dependent [93]. Considering structural similarities of phytoestrogens to estradiol and their ER-mediated actions, the ovary is undoubtedly a potential target organ for the actions of phytoestrogens. As shown by others, phytoestrogens generally possess a greater affinity for ERβ than ERα [94; 95]. Not only is it the ovary is a main site of steroidogenesis in females, also ERβ expression has been found to be the highest in ovarian granulosa cells [96; 97]. This part of discussion will outline the *in vitro* effects of phytoestrogens at ovarian cells.

3.1 Steroidogenesis disruption by phytoestrogens

A growing body of evidence from *in vivo* animal and human studies and *in vitro* studies supports the notion that phytoestrogens can affect ovarian steroidogenesis in females, which further results in deregulation of the reproductive axis. Phytoestrogens were shown to compete with endogenous substrates for active sites of steroidogenic and metabolizing enzymes. Also, phytoestrogens stimulate hepatic synthesis of sex hormone binding globulin (SHBG), thereby indirectly reducing the amount of free, biologically active E2 in the serum [98]. There are however conflicting results with regard to the effects of phytoestrogens on circulating E2 levels in females. The studies on rats reported either lack of changes [99; 100] or increased E2 levels in ovariectomized rats after isoflavone administration [101]. However, in pre-menopausal women, the consumption of isoflavone-rich soy foods decreased the circulating levels of E2 and led to the attenuation of pre-ovulatory surge of LH

and FSH [102; 103; 104]. However, others showed that exposure to isoflavones has no impact on circulating levels of ovarian hormones [105; 106].
The complexity of follicular steroidogenesis makes it difficult to study it *in vivo*. This process involves a cascade of multiple enzymes, of which each one affects precursor supply to another. One important rate limiting step in steroidogenesis is steroid acute regulatory protein (StAR), which is involved in the translocation of cholesterol from the outer to the inner mitochondrial membrane [107; 108]. Studies with StaR knockout female mice demonstrated impaired follicular maturation manifesting in anovulatory stage of the ovaries, which were lacking corpora lutea [109]. As yet, there is only limited data on phytoestrogen-mediated effects on StAR gene and protein expression in steroid hormone producing cells. An indication on modulatory effects of diverse phytoestrogens on *StAR* mRNA and protein comes from studies with the *in vitro* MA-10 murine Leydig cells [110]. In this study, the StAR promoter activity was shown to be inhibited after exposure to genistein (GEN) or resveratrol (RESV) at concentrations higher than 10 or 25 µM, respectively. In contrast, quercetin (QUE) at 25µM enhanced StAR promoter activity, presumably through the increase of Ca^{2+} influx via voltage-gated Ca^{2+} channels [110]. In our study with human granulosa KGN cells, *StAR* mRNA levels were also increased by QUE, coumestrol (COU), RSV, and 8-prenylnaringenin (8-PN) but not by GEN (Chapter 4). Furthermore, the weakly estrogenic Naringenin (NAR) did not have an effect on *StAR* mRNA expression in both KGN cells and porcine oocytes (Chapter 4 and 5). Taken together, these *in vitro* results clearly demonstrate that StAR can be a potential biological target of phytoestrogen modulation of ovarian steroidogenesis. However, it is important to stress, that steroidogenesis is driven by a hormonal modulators such as LH and FSH and other autocrine and/or paracrine factors, which all were shown to stimulate *StAR* mRNA levels and augment the steroid hormone production. These crucial hormonal feedback mechanisms are usually not taken into consideration in *in vitro* experiments. Thus, for better prediction of phytoestrogen effects on steroidogenic enzymes i.e. StAR modulation, future *in vitro* studies should include the co-exposure of phytoestrogens with natural hormones.

While assessing the phytoestrogen action on ovarian steroidogenesis, our main emphasis was directed towards aromatase (CYP19A1) modulation by these compounds. As described in Chapter 4, CYP19A1 plays a key role in the final step of steroidogenesis by converting androgens into estrogens. When investigating the genomic action of phytoestrogens on CYP19A1, it is crucial to assess the action of phytoestrogens on aromatase promoters, since its expression is regulated via different promoter regions in a tissue-specific manner [111]. The ovarian *CYP19A1* expression is driven by promoter II (PII) and further modulated by gonadotropins and cyclic-AMP dependent signaling pathways [112]. In KGN cells, however, both PII and P I.3 are active (Chapter 4). In chapter 4 we showed that phytoestrogens such as 8-PN, GEN, QUE up-regulated *PII* and *P1.3*-driven aromatase levels. On the other hand, other *in vitro* studies indicated that phytoestrogens were able to inhibit CYP19A1 activity and its gene expression in other types of tissue [113]. Inhibitory effects on aromatase activity were also observed by us as described in

chapter 4. Interestingly, 8-PN was shown to concentration-dependently inhibit *in vitro* aromatase activity, with an IC_{50} that was similar to the therapeutic aromatase inhibitor letrozole (Chapter 4). Future studies with 8-PN are needed to evaluate if this potent estrogenic compound inhibits aromatase also *in vivo*. Moreover, more recent studies have highlighted the cross-talk of both genomic and non-genomic action of EDCs including phytoestrogens [114]. In the view of the above results, future studies should be also focus on membrane-initiated steroid signaling pathways e.g the fast responding kinase pathways, which may be the prerequisite for subsequent genomic responses [115]. This integration of membrane-initiated effects with genomic mechanisms may improve our knowledge on the cellular mechanisms and responses occurring after exposure to these endocrine modulating compounds.

As indicated by others, exposure to phytoestrogens can result in the significant estrogenic stimulation, which may lead to tumor formation in some tissues [116]. Upon exposure to GEN and daidzein, *in vitro* and *in vivo* studies have shown for instance an increase of proliferation and DNA damage in hormone-sensitive MCF-7 breast cancer cell lines [117], induction of particular type of cancers, such as the uterine adenocarcinoma and vulvar carcinomas in mice [118; 119] or colon cancer in rats [120]. Yet, the possible beneficial or adverse effects of phytoestrogens on estrogen sensitive tumors in humans are not equivocal. Nevertheless, some epidemiological studies, mainly in Asian populations, support a notion of lower occurrence of several cancers including breast, prostate and colon cancer in populations consuming a high soy-based diet.

With respect to tumor initiation process, the crucial step of metastasis involves the invasion of cancer cells into surrounding tissue and the start of neovascularization process [121]. The data obtained from *in vitro* and *in vivo* animal studies indicate, that phytoestrogens may influence the carcinogenic process by diverse modulations of cell function, independently of their antioxidant properties [122; 123]. For example, it has been reported, that RSV and QUE inhibited the migration of vascular endothelial cells in a concentration-dependent manner with IC50 values of 18.8 μM and 37.5 μM, respectively [122]. Similarly, in our studies RSV and QUE were shown to cease the migration of KGN cells, with the most pronounced effects observed in GEN-treated cells [124] (Chapter 4). Strikingly, in our study, phytoestrogens did not affect KGN cell proliferation, nor cell cycle distribution. Others have shown that phytoestrogens such as GEN, daidzein, and equol were able to modulate proliferation of estrogen-responsive cells such as breast cancer cell lines [125; 126]. At low concentrations (1 to 100 nM), GEN was shown to inhibit proliferation of Ishikawa cells, an epithelial cell line derived from human endometrial adenocarcinoma, in a concentration-dependent manner [127]. In contrast, similar concentrations of GEN promoted the proliferation of uterine leiomyoma cells [128]. This implicates that the effects by which phytoestrogens affect proliferation of cancer cell lines is tissue-dependent and their responses may vary depending on different nuclear receptor content e.g. the presence or absence of ERα or ERβ.

The ovary is responsible for the promotion of growth of pre-ovulatory follicle(s). It is also well documented that the regulation of each component of the follicle can be affected by EDCs and its modulation can have adverse consequences on the reproductive outcome. In case of the exposure to phytoestrogens, disrupting effects on the follicle can result in a decrease of reproduction capacity in farm animals. For instance, cattle fed with red clovers containing a rich source of isoflavones, developed ovarian cysts [129]. Also, a massive number of small and medium-sized follicles, and follicle atresia was observed after treatment of ewes with this plant [130; 131]. In addition, a lower number of Graafian follicles with the absence of corpus luteum, has been detected after the neonatal exposure to COU in mice [132].
The oocyte is for its growth and maturation dependent on the proper health and development of the surrounding somatic cells. These include granulosa cells, which change morphology and proliferate when an oocyte begins the process of folliculogenesis. This oocyte–cumulus cell interaction provides an appropriate micro-environment that nurtures oocyte cytoplasmic maturation and has signifcant impact on the developmental competence of the embryo [133]. *In vitro*, some phytoestrogens exert negative effects on the growth of cumulus-oocyte complexes. For instance, GEN concentration-dependently inhibits the cumulus cell expansion [134]. Similarly, NAR and 8-PN caused abnormal oocyte maturation by negatively affecting the cumulus-oocyte complex (Chapter 5), [135]. However, apoptosis or changes in expression of pentraxin 3 (PTX3) could explain this adverse outcome [135]. Conversely, supplementation of the maturation medium with QUE significantly increased the rate of cumulus cell expansion, which resulted in a significant improvement of embryonic development and blastocyst ratio [136; 137]. Obviously, the quality of the follicular environment in which the oocyte develops, impacts the nucleus maturation. In our study (Chapter 5), both NAR and 8-PN severely affected spindle formation. In turn, this impairment of the nuclear maturation resulted in the decrease of the oocyte cleavage and blastocyst formation (Chapter 5). Together, the *in vitro* results on cumulus-oocytes complexes treated by phytoestrogens support the occurrence of adverse effects of these plant-derived compounds on female reproductive factors. This result correlates with *in vivo* effects, as described earlier. Further research utilizing porcine oocytes and phytoestrogens or other EDCs can provide more insight, to which extent these compounds may modulate the crucial developmental processes in follicle and embryonic stage.

PART III. Future directions for non-animal testing within HPO-axis

Without doubt, chemicals must be tested for their adverse effects on human reproduction. However, reproductive toxicity testing, as it is performed according to the present animal model protocols, is one of the most time, animal and resource intensive toxicity tests [138]. As explained in the introduction, reproductive toxicity testing requires more than 2.8 million animals out of the estimated 4 million animals to be used for safety testing under the REACH legislation [138]. Some attempts

have already been made to reduce the number of animals for reproductive and developmental toxicity testing. For instance, an accepted OECD guideline for an extended one-generation study (OECD TG 443) could reduce animal use by 40% comparing to the currently used 2-generation study (OECD TG 416). This adaptation could lead to a decrease of approximately 15% of animals that are going to be used in the entire REACH program [139; 140].
The above mentioned reproductive toxicity tests are used to determine adverse effects on fertility, growth and development of the foetus, and, in case of the two-generation study, functional assessment of fertility after a complete reproductive cycle exposure [141]. However, the mechanism by which possible adverse reproductive outcome occurs, can normally not be established using these animal-based toxicity tests. For instance, the modulation of the GnRH neuronal network by dioxin-like chemicals leading to neuro-endocrine alterations, as investigated in Chapter 3, would remain unidentified. This 'black-box' approach in animal toxicity testing may hamper the extrapolation of both the effective dose and specific effect to humans, as species differences exist with respect to e.g. the cellular and species specific differences in steroidogenic enzyme expression [28; 142]. The *in vivo* uterotrophic assay (OECD 440), commonly used to asses (anti-)estrogenic properties of chemicals also has its limitations with respect to prediction of human risks, because the responses to estrogenic and especially anti-estrogenic compounds can be species-dependent [143]. This is clearly illustrated by tamoxifen, which is mainly an estrogen antagonist in the rat uterus, but an agonist in the human, mouse, guinea pig, and dog uterus [144]. With respect to validated *in vitro* methds, some OECD-accepted *in vitro* tests exist, such as the embryonic stem cell test (EST), micromass embryotoxicity assay or the whole rat embryo embryotoxicity assay. These tests are focused on emryotoxicity and only cover a limited critical period of mammalian reproductive development.
Thus, the correct interpretation of experimental data requires awareness of the fact that some endocrine modulators, including phytoestrogens, can induce different effects depending on dose, tissue or species [145]. Furthermore, understanding the difference in effects on HPO- axis regulation between high and low doses is also crucial with respect to exposure of e.g. EDCs. Exposure to phytoestrogens can result in non-monotonic responses, such as the observed biphasic effects of QUE and 8-PN on aromatase activity (Chapter 4) and spindle formation (Chapter 5) in our studies. The lack of knowledge and relevance of this mechanism across species impede the risk assessment of low dose human exposure to EDCs. Moreover, the lack of proper biokinetic mechanisms in *in vitro* models hampers the assessment of its relevance with respect to possible adverse reproductive outcome pathways in humans.

As elaborated in PART I, a key advantage of *in vitro* cell models of human origin is that the information on the mechanism of toxicity of endocrine-modulating compounds can be explicitly obtained for human relevant effects and endpoints. For instance, as described in PART I, KGN cells are human-derived cells with a relatively high aromatase activity and could therefore potentially served as a model to assess effects of EDCs on ovarian steroidogenesis. Nevertheless, the greatest difficulties of *in vitro* cell systems still lies in the extrapolation of *in vitro* findings to the *in vivo*

situation, because it remains difficult to translate results from *in vitro* results to an adverse outcome in an intact organism. As stated earlier, the use of single cell-type cultures for evaluation of toxicity with respect to the HPO-axis clearly has its restrictions. Such *in vitro* systems lack the important interactions between different cell types within an organ or between multiple organs (as elaborated in PART I). Therefore, identification of EDCs-targeted cells and/or organs using *in vitro* single cell line models may give only a fragmentary picture, which could miss endocrine perturbations occurring elsewhere in the body.
One of the possibilities to increase the realism of *in vitro* systems can be the application of combined cell systems in so called co-culture models. At present, several of these co-culture models have been introduced for toxicity testing and provide possibilities to study cell-cell interactions. In addition, xenobiotic toxicity can also be assessed in the presence of multiple cell types, named the Integrated Discrete Multipleorgan Co-culture (IdMOC), which may include metabolically competent cells [146; 147; 148]. However, it should be realized that none of the present co-culture models is yet suitable to cover the full reproductive cycle in females. As mentioned in PART I, several different cell lines are already available that allow the endocrinologist and toxicologist to study specific single endocrine modulatory effects of EDCs. Yet, their applications are limited for the toxicological risk assessment at regulatory level. This is mainly caused by the poor ability of these *in vitro* models to replicate the complex biochemical, molecular and functional interactions, including feed-back loops that are occurring *in vivo* in the HPG-axis [149; 150]. Moreover, the obtained results in such mono- or co-culture systems does not easily translate into an equivalent human oral dose, which may be associated with an adverse reproductive outcome. In this respect, biokinetics factors like absorption, distribution, metabolism and excretion (ADME) also play a significant role in creating this uncertainty for extrapolation of *in vitro* data to the *in vivo* situation. [151; 152; 153; 154]. In conclusion, only limited data can be obtained from the current cell culture systems with respect to reproductive impairment that can be used directly for human risk assessment [155; 156].

A way around the issue of complex kinetic and dynamic processes determining the effect of EDCs on HPO-axis maybe the integration of a battery of *in vitro* assays, which mimics the various hormonal feedback mechanisms within HPO-axis. Such a strategy for human risk assessment is referred to as integrated testing strategy (ITS) and adverse outcome pathway (AOP) modeling [157; 158]. Within the OECD, such an ITS approach is considered and currently drafted in the OECD Conceptual Framework (CF, OECD Work Related to Endocrine Disrupters). In essence, the OECD conceptual framework is an outline of an *Intelligent (or Integrated) Testing Strategy* (*ITS*). It is involving *in vitro* battery testing in chemical risk assessment and has been reviewed by, amongst others, [157; 159]. These strategies include, approximately in order of first use, thresholds of toxicological concern (TTC), *in silico* read across studies and (quantitative) structure-activity relationships (QSARs), *in vitro* kinetics (e.g. human cell-based intrinsic clearance assays) and dynamic assays (e.g. omics approaches, porcine oocyte nuclear maturation assays, Chapter 5), *in silico* systems biology approaches and physiologically based biokinetic and toxicodynamic modeling (PBBK/

PBPD), or finally *in vivo* studies on lower organisms and human volunteer and clinical studies [159; 160].
In addition, another framework AOP, launched by the OECD in 2012, has been introduced [161]. This tool was introduced to capture the series of key events starting from an interaction of a chemical with a biological target (the molecular-initiating event (MIE)) e.g. binding to receptor protein. This step initiates a change at the cellular and molecular level, which subsequently can lead to macroscopic effects at tissue and/or organ level. Finally, this sequence of events may result in an adverse outcome for the individual organism that may effect a whole population. The OECD has pointed out that such an AOP approach can allow profiling of EDCs according to their potency to trigger adverse outcomes, instead of associating those compounds with the final adverse effect only (see Fig. 1)

Given the complexity how the HPO-axis is regulated and modulated via different molecular signaling pathways, the use of ITS strategies and AOP models in human risk assessment of EDCs in the near future seems inconceivable. Nonetheless, it is worth focusing research recourses on the establishment of such strategies and models.

Up till now, several computational models of the HPG axis have been developed [162; 163; 164; 165; 166]. Most of those models focus on the endocrine modulation using fish as a target model. These models are especially being used to predict changes in plasma sex-steroid concentrations in the presence of e.g. PCBs and cadmium in female and male fish [162; 163; 164; 165].
However, some *in silico* models for the mammalian HPG axis also exist. Barton and Andersen (1998) developed a model of the HPG axis in the rat to simulate hormone levels in testes and blood. Recently, Quignot and Bois (2012) published a computational model of the last steps of steroidogenesis in the rat ovary to predict sex steroid levels using *in vitro* data from primary rat granulosa cell cultures [166]. In addition, PBBK/PBPD models of the HPG axis have been developed for humans [167]. One of these models specifically describes changes in LH and T concentrations following treatment with the GnRH agonist triptorelin and the GnRH receptor blocker degarelix [167]. Up till now there are no validated PBBK/PBPD models of the HPG axis set for testing endocrine modulation.
For a future approaches, we propose to develop an integrated PBBK/PBPD of the HPG axis for EDCs testing based on available *in vivo* (animal and human) and *in vitro* data. This model will provide the quantitative tools to translate *in vitro/ in vivo* results into predictions of outcomes at the level of the organism. Such a PBBK/PBPD model must clearly contain the information on homeostatic control and activity of hormones and hormonal feedback mechanism pathways based on the quantitative *in vivo* predictions. In such a model the molecular targets and the cellular, tissue, and organ outcomes that occur in response to chemical exposure shall preferable be incorporated on the basis of the *in vitro* measurements using for instance the reverse dosimetry [152; 168; 169]. This reverse dosimetry approach can provide a translation of *in vitro* effect concentration (e.g. an EC50 value) into a single *in vivo* effective dose

(e.g. a lowest observed adverse effect level (LOAEL)) [170; 171; 172]. Also, reverse dosimetry can serve to estimate the external exposure that would result in effective concentrations at relevant tissue and /or organ targets [168]. The ultimate outcome of such an approach would be to establish a wide-ranging computational HPG axis model that would define key biological events occurring within HPO-axis for toxicity endpoints.

To summarize, for the replacement of animal use in toxicological risk assessment, reliable *in vitro* and *in silico* assays as alternatives for *in vivo* reproductive toxicity studies are highly needed. In addition, the integration of *in vitro* toxicity assays with *in silico* models with respect to the HPO-axis may serve as potential tool to support the reduction of animal use for reproductive and developmental toxicity tests. Such a framework provides a vision for the evaluation of chemical-induced endocrine modulation by focusing on their mechanisms of action and by utilizing the validated *in vivo* and *in vitro* methods. Additionally, these models may serve as valuable tools to interpret scientific data and to further predict adverse responses for varied human exposure situations.

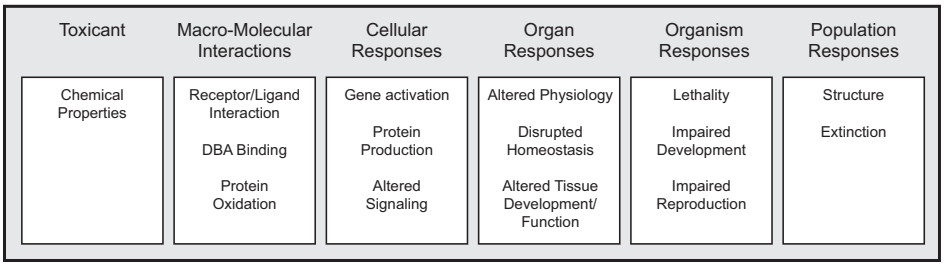

Fig.1 A schematic representation of an Adverse Outcome Pathway (AOP). Adapted from OCED http://*www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm*

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Nederlandse Samenvatting

Introductie

Een belangrijke functie van voortplanting is het voortbestaan van de soort. De vrouwelijke cyclus is een van de meeste ingewikkelde hormonale processen die plaatsvindt in zoogdieren. Het wordt gereguleerd door een nauwkeurig netwerk van neurologische en hormonale signalen van de hypothalamus, de eierstokken en de hypofyse. Voor onderzoek naar de effecten van hormoonverstorende stoffen is de Hypothalamus- Hypofyse- Gonade- as (HHG) van bijzonder belang omdat er sterke aanwijzingen zijn dat sommige hormoonverstorende stoffen de normale aansturing en werking van dit dynamische systeem beïnvloeden. Bij vrouwen kan dit leiden tot veranderingen in de hormonale cyclus en heeft het invloed op de vrouwelijke seksuele ontwikkeling en werking. De nadelige effecten van de hormoonverstorende stoffen op het hormonale systeem zijn voornamelijk toe te schrijven aan de oestrogene en/of anti-oestrogene aard van deze stoffen. Het mechanisme van de hormoonverstoring betreft de verandering in de verschillende cellulaire mechanismen waarbij de oestrogeenreceptor (ER) en de Aryl-koolwaterstof-receptor (AhR) de cellulaire reacties in de voortplantingsorganen beïnvloeden. De vele dierstudies en epidemiologische gegevens uit humaan onderzoek over dioxinen en PCB's hebben aangetoond dat bij onderzochte diersoorten de blootstelling aan deze verbindingen een groot aantal negatieve effecten op de seksuele ontwikkeling en de fysiologie van het voortplantingsstelsel kan veroorzaken. Fyto-oestrogenen vertonen een structurele gelijkenis met oestrogenen en kunnen zorgen voor zowel oestrogene en anti-oestrogene effecten, afhankelijk van de concentratie van lichaamseigen oestrogenen en oestrogeenreceptoren. Deze groep van verbindingen beïnvloedt de humane oestrogeenbiosynthese en de menstruele cyclus. Hoewel er veel *in vivo* en *in vitro* testmethoden ontwikkeld zijn voor het screenen van hormoonverstorende stoffen is de kennis nog beperkt over hoe hormonale veranderingen binnen de Hypothalamus-Hypofyse-eierstok (HHE)-as kunnen optreden. In de voortplanting werken veel verschillende cel- en orgaansystemen samen. De gegevens uit humane studies zijn te beperkt en de toxische effecten van hormoonverstorende stoffen worden nu voornamelijk *in vivo* onderzocht. Deze dierstudies zijn, zowel in tijd als in geld, erg kostbaar en vanuit een ethisch oogpunt discutabel. Er is dus een grote behoefte aan de verdere ontwikkeling van *in vitro* onderzoeksmethoden op dit gebied.

2. Doel

Dit proefschrift heeft twee hoofddoelen:

1. De ontwikkeling van *in vitro* modellen om de hormoonverstorende werking in de hypothalamus-hypofyse-eierstokken (HHE)-as te onderzoeken

2. Een *in vitro* evaluatie van het mechanisme achter de hormoonverstorende effecten van bekende hormoonverstorende stoffen (zoals dioxines en fyto-oestrogenen) op de hypothalamus, de hypofyse en de eierstokken.

Om deze doelen te bereiken is dit proefschrift opgedeeld in vier verschillende onderzoeken. De belangrijkste uitkomsten worden hieronder beschreven.

Hoofdstuk 2 beschrijft de toepasbaarheid van het gebruik van primaire celkweken afkomstig van de hypothalamus, de hypofyse en de gonaden van ratten voor onderzoek naar de nadelige effecten van dioxine (TCDD) en structureel vergelijkbare niet-dioxine-achtige verbindingen op de HHE. Er is onderzocht waar in de HHE-as effecten van TCDD, dioxine-achtige (PCB126) en niet dioxine-achtige (PCB153) optreden en of deze *in vitro* celreacties de *in vivo* resultaten kunnen voorspellen. De uitkomsten van dit onderzoek zijn in overeenstemming met de beschikbare *in vivo* gegevens van de effecten dioxine-achtige stoffen op het voortplantingssysteem. Daarnaast geeft **Hoofdstuk 2** informatie over de mechanistische werking van deze toxische stoffen op het HHE-systeem. De resultaten tonen aan dat de AhR-gemedieerde effecten van dioxines tot verstoringen kunnen leiden in alle getest *in vitro*systemen, waarbij de meest sterke effecten optreden op het niveau van de eierstokken. In de hersenen bleken Kiss-1 neuronen in de hypothalamus doelwit van dioxinen en niet-dioxine-achtige verbindingen. In de hypofyse en de eierstokken werd voornamelijk het FSH-mechanisme verstoord door dioxine-achtige stoffen. De significante veranderingen in de genexpressie van Cyp17 en Cyp19A1 door dioxine-achtige stoffen duidt op een mogelijk direct, AhR-gestuurde effect op enzymen die zorgen voor geslachtshormoonproductie in de ovaria. Om de mechanistische basis van de dioxine-achtige werking beter te begrijpen kan je d.m.v. klein interfererend RNA (siRNA) de AhR uitgeschakelen. Deze techniek wordt beschreven in **hoofdstuk 3.** Het is bekend dat AhR betrokken is bij veel biologische en toxicologisch reacties, waaronder de neuro-hormonale signalering. Op dit moment ontbreekt een geschikt *in vitro* model om de mogelijke effecten van lichaamsvreemde verbindingen op cellulair en moleculair niveau in de hypothalamus te onderzoeken. Een hypothalamus model of cellijn zou idealiter de AhR-pathway moeten bevatten. In dit onderzoek is gebruik gemaakt van een nieuwe GnV-3 cellijn afkomstig uit de hypothalamus van de rat. Deze cellijn brengt de AhR tot expressie en is dus ogenschijnlijk een goed model voor de AhR-gereguleerde neuro-hormonale verstoring in de hypothalamus. **Hoofdstuk 3** beschrijft een aantal belangrijke resultaten die tonen dat een aantal AhR-afhankelijke reacties overeenkomt met het circadiaan ritme (biologisch ritme) en de voedselinname maar niet met aansturing van de serotonine receptor (5-HT). Daarnaast toont deze studie aan dat de GnV-

3 cellen ongeveer een 24-uurs circadiaans ritme hebben, vergelijkbaar met het patroon van Gnrh genexpressie. We kunnen dus concluderen dat de GnV-3 cellijn kan worden beschouwd als een aanvulling op bestaande methoden voor toekomstig onderzoek naar de neuro-hormonale effecten van andere AhR-verbindingen op de hypothalamus. Er is wetenschappelijke zorg gerezen over de mogelijk negatieve effecten van fyto-oestrogenen op de vrouwelijke voortplanting. Deze van planten afgeleide oestrogene verbindingen worden in toenemende mate gebruikt vanwege de gesuggereerde gezondheidsbevorderende eigenschappen, zelfs door jonge gezonde vrouwen. Het onderzoek zoals beschreven in **hoofdstuk 4** werd uitgevoerd met granulosa KGN cellen afgeleid van humane kankercellen. Hiervoor is eerder aangetoond dat deze een perfect *in vitro* model zijn voor het bestuderen van effecten op de werking van humane granulosa cellen. De resultaten van dit onderzoek tonen aan dat fyto-oestrogenen van invloed kunnen zijn op verschillende reacties in granulosa-achtige cellen door een verandering van StAR genexpressie en de wijziging van ovaria-specifieke promotor II en I.3 aromatase expressie en activiteit. Ook verminderen de meeste van de geteste fyto-oestrogenen de KGN-celmigratie, maar het moleculaire mechanisme hierachter moet nog verder worden onderzocht. KGN-cellen blijken een waardevol model te zijn voor het bestuderen van de effecten van hormoonverstorende stoffen in de eierstokken. In **hoofdstuk 5** wordt het onderzoek naar de nadelige effecten van fyto-oestrogenen op het voortplantingssysteem in de eierstokken voortgezet. **Hoofdstuk 5** benoemt een goed bruikbaar en veelbelovend *in vitro* model met primaire eicellen van varkens voor toxicologisch voortplantingsonderzoek. De effecten van de flavononen naringenin (NAR) en 8-prenylnaringenin (8-PN) worden beschreven. Dit onderzoek toont aan dat NAR en 8-PN een abnormale eicelrijping veroorzaken vanwege het effect op het cumulus-eicel-complex en het aantasten van de *in vitro* ontwikkelingspotentie van de eicellen. De blootstelling van eicellen aan NAR veroorzaakte een concentratie-afhankelijke daling van de spindelvorming. 8-PN lijkt een tweefase effect te hebben op de spindelvorming. Belangrijker is dat de blootstelling aan NAR en 8-PN leidde tot een afname van de eicelsplitsing en kiemcelvorming bij fyto-oestrogeenniveaus die in oestrogene activiteit vergelijkbaar zijn met fysiologische oestradiol-niveaus in varkens follikels. Opvallend was de concentratie-afhankelijke opstapeling en fuseren van vetdruppels in varkens-eicellen na behandeling met NAR. Ondanks de gebrekkige farmacokinetische kennis van fyto-oestrogenen, is in **hoofdstuk 4** en **5** aangetoond dat deze stoffen een effect kunnen hebben op de eierstokwerking en eicelrijping bij concentraties die gevonden worden in plasma na de inname van supplementen. Deze resultaten wijzen erop dat overvloedige inname van fyto-oestrogenen bijvoorbeeld via voedselsupplementen vermeden zouden moeten worden door vrouwen. In deel 3 van de discussie worden de toekomstperspectieven van alternatieven voor dierproeven en *in vitro* testen voor voortplantingstoxiciteit beschreven. De nadruk is gelegd op de ontwikkeling van 'integrated intelligent testing strategies" voor het onderzoek naar hormoonverstorende stoffen. Dit hoofdstuk beschrijft de toepasbaarheid van een geïntegreerd PBPK/PBPD computermodel van de HHG-as voor onderzoek naar hormoonverstorende stoffen. Dit kan worden bereikt door ingewikkelde concentratie en tijds-afhankelijke relaties en receptor interacties in

de HHE-as beter te voorspellen met behulp van integratie van gegevens uit *in vivo* dierexperimenteel onderzoek, *in vitro* onderzoek waarbij vervolgens de *in vitro* dosis wordt omgerekend naar een *in vivo* blootstelling, en humaan klinische gegevens. De gegevens uit een dergelijk model kunnen gebruikt worden als instrument voor de verbetering van het begrip van het dynamische gedrag in deze as en het beter extrapoleren van de resultaten van *in vivo* en *in vitro* humaan onderzoek. Tevens kan het geïntegreerde PBPK/PBPD model gebruikt worden voor de vroege identificatie van mogelijke moleculaire biomarkers in de wettelijk verplichte veiligheidstoetsing van hormoonverstorende stoffen.

Ciriculum Vitae

Kamila Solak was born in Krakow, Poland on the 17th of January, 1980. After completing high school, she commenced her five-year Master's degree studies in Biology at Jagiellonian University in Krakow. During her Master programme she was working on biomarkers of environmental exposure in human placenta. She obtained her Master of Science degree in 2004. In 2006 she completed one year of postgraduate studies in Industrial Pharmacy, Collegium Medicum in Krakow. Her thesis focused on Food and Drug Administration (FDA) and regulations related to placing medicinal products on the US market.

In years 2005-2008 she was working for the National Health Fund in Krakow, Poland. During this time (2006-2008) she continued to develop her research interest by getting involved in work carried out at the Department of the Experimental Hematology at Jagiellonian University, Krakow, Poland. In addition to research, her responsibilities involved teaching an Animal Physiology course to university students.

In 2009 she completed an Environmental Cancer Risk Nutrition and Individual Susceptibility (ECNIS) Exchange Fellowship at the National Hellenic Research Foundation, Athens, Greece. The project focused on the investigation and exploration how endocrine modulators of environmental or nutritional origin may affect breast cancer risk through regulation of Aryl-hydrocarbon receptor (AhR) signaling. At the end of 2009 she moved to Utrecht, the Netherlands and started her PhD project on the effects of Endocrine Disrupting Compounds (EDCs) on Hypothalamus-Pituitary-Ovary (HPO)-axis *in vitro* at the Institute for Risk Assessment Sciences (IRAS), Utrecht University with the overall objective of elucidating the *in vitro* methods for endocrine modulation within the reproductive axis. Kamila is currently employed as an European EHS specialist in Brussels, Belgium.

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