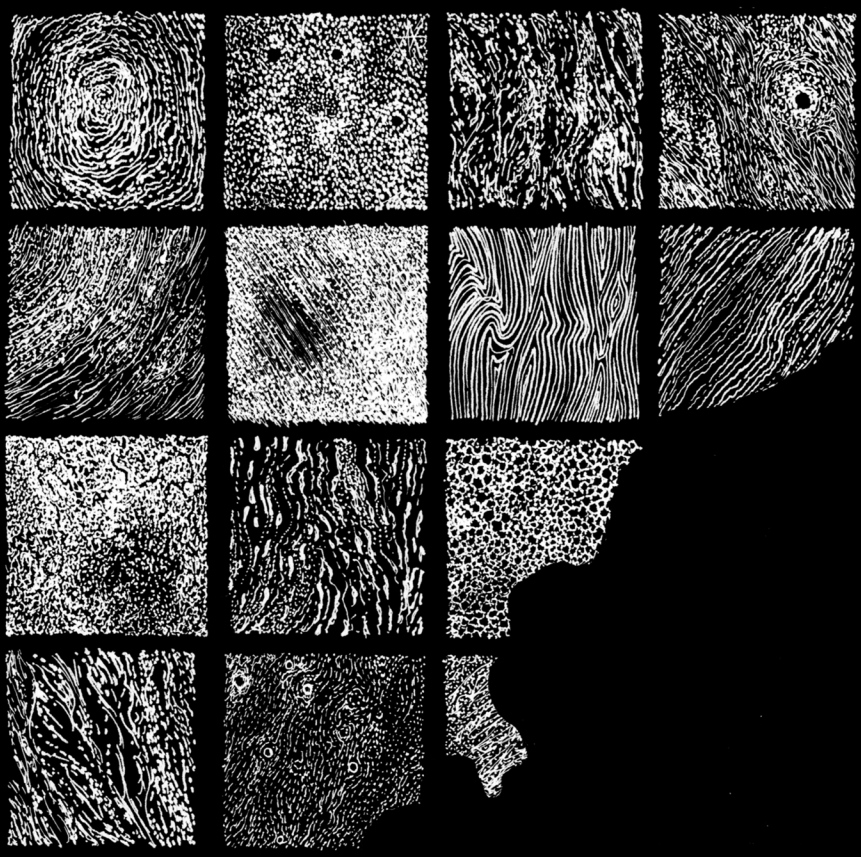
DIRECT, MULTI- AND TRANSGENERATIONAL EFFECTS OF ENDOCRINE DISRUPTORS ON MATERNAL BEHAVIOR AND FEMALE SEXUAL MATURATION

By David López Rodríguez



A dissertation submitted for the degree of Doctor of Psychology

GIGA-Neurosciences
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SEPTEMBER 2020

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DIRECT, MULTI- AND TRANSGENERATIONAL EFFECTS OF ENDOCRINE DISRUPTORS ON MATERNAL BEHAVIOR AND FEMALE SEXUAL MATURATION

Abstract

By David López Rodríguez
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Our society is facing a public health challenge due to the increasing presence of Endocrine Disrupting Chemicals (EDCs) in the environment. Developmental exposure to EDCs, by interacting with endogenous hormonal pathways, disrupts the organization of the central nervous system leading to life long-lasting consequences. Exposure during such critical period of development may interfere with germ cell development, affecting th next generations.

Our goal was (1) to study the effects of developmental or adult exposure to low doses of BPA on the neuroendocrine control of reproduction in females; and (2) to study the effects of exposure to a low dose EDC mixture on sexual maturation and maternal care across generations. We have shown that adult BPA exposure transiently disrupts estrous cyclicity and ovarian follicle development by affecting the preovulatory LH surge. Perinatal exposure to EDC mixture transgenerationally alters sexual maturation and reproductive function throughout epigenetic reprogramming of the neural network controlling GnRH secretion. Such exposure also induces a multigenerational loss of dopamine signaling and maternal care. A cross-fostering paradigm indicated a potential germline transmission explaining the transgenerational reproductive phenotype.

Overall, we identified alterations of the hypothalamic control of reproduction and maternal behavior in female rats up to the fourth generation after EDC exposure. These results raise concerns about the legacy we give to future generations and urge to further evaluate the effects of EDCs across generations in human populations.

EFFETS DIRECTS, MULTI- ET TRANSGENERATIONNELS DE PERTURBATEURS ENDOCRINIENS SUR LE COMPORTEMENT MATERNEL ET LA MATURATION SEXUELLE

Résumé

Notre société fait face à un problème grandissant de santé publique lié à la présence croissante des perturbateurs endocriniens (EDCs) dans notre environnement. Une exposition développementale peut entrainer des altération dans l'organisation du cerveau et avoir des conséquences à long terme sur la santé. L'exposition pendant cette période critique peut ainsi affecter le développement et la différenciation des cellules germinales affectant plusieurs générations.

Ce travail vise à déterminer (1) l'effet du bisphénol A (BPA) après une exposition développementale ou adulte sur le système neuroendocrinien contrôlant la reproduction chez le rat femelle; et (2) l'effet d'un mélange d'EDCs sur la maturation sexuelle et le comportement maternel à travers plusieurs générations. Ce travail a révélé qu'une exposition adulte au BPA altère transitoirement la folliculogenèse et le contrôle neuroendocrine de l'ovulation. L'exposition au mélange d'EDCs altère transgénérationellement le système reproductif au niveau central et périphérique à travers une reprogrammation épigénétique de l'hypothalamus. Ces effets pourront être causés par une altération des cellules germinales. En outre, nous avons mis en évidence une diminution multigénérationnelle de la signalisation dopaminergique hypothalamique et du comportement maternel.

Globalement, nous avons identifié des altérations reproductives et comportementales pendant 4 générations d'animaux après une exposition aux EDCs ainsi que leurs mécanismes associés au niveau hypothalamique. Ces résultats soulèvent des inquiétudes sur les conséquences potentielles des EDCs sur les générations futures chez l'humain.

Par David López Rodríguez
Université de Liège

EFECTOS DIRECTOS, MULTI- Y TRANSGENERACIONALES DE LOS DISRUPTORES ENDOCRINOS SOBRE EL COMPORTAMIENTO MATERNO Y LA MADURACION SEXUAL

Resumen

Por David López Rodríguez
Universidad de Liège

Nuestra sociedad se enfrenta a un gran desafío debido al aumento de disruptores endocrinos (EDCs) en el medioambiente. Una exposición durante el desarrollo a los EDCs, a través de su interacción con lon las hormonas endógenas, altera la organización del sistema nervioso central y puede tener consecuencias durante toda la vida. La exposición durante este periodo tan crítico del desarrollo puede interferir con las células germinales, afectando así a generaciones futuras.

El objetivo de esta tesis es de estudiar el efecto de bajas dosis (1) de bisfenol A (BPA) tras una exposición neonatal o adulta en el eje reproductivo; (2) de una mezcla de EDCs en la maduración sexual y el comportamiento materno a través de cuatro generaciones. Los resultados muestran que, a la edad adulta, el BPA altera transitoriamente la foliculogenesis y el control neuroendocrino de la ovulación. La mezcla de EDCs altera transgeneracionalmente el sistema reproductivo a nivel central y periférico, reprogramando epigenéticamente el sistema neuroendocrino. Dicha exposición disminuye, además,la señalización dopaminérgica hipotalámica y el comportamiento materno multigeneracionalmente. Un experimento de interadopción reveló que el fenotipo reproductivo podría deberse a alteraciones en las células germinales.

En conjunto, hemos identificado alteraciones en el control hipotalámico de la reproducción y el comportamiento materno, presentes hasta cuatro generaciones de ratas hembras tras la exposición a una mezcla de EDCs. Estos resultados suscitan inquietud sobre el legado que damos a generaciones futuras y urge a las agencias de regulación a aplicar el principio de precaución.

Foreword

Virtually everyone, in developed and developing nations, is exposed to dozens -if not hundreds- of chemicals on a daily basis. The environment (i.e. air, soil, water supply), common consumer goods (i.e. packaging, personal care products) and food sources contain pollutants called Endocrine Disrupting Chemicals (EDCs) that are able to alter the normal function of the endocrine system and cause adverse effects in human health. Persistent EDCs accumulate in the environment as well as in wildlife and human fat tissue. The increasing presence of these chemicals during the last centuries pose a significant concern for public health that urges scientist to study their adverse effects and mechanisms of action.

There is a large variety of EDCs including drugs, pesticides and fungicides, plasticizers, industrial by-products and some naturally occurring botanical chemicals. These compounds have been related to lower IQ in children, cancer development, behavioral, metabolic and neurodevelopmental disorders, obesity and male and female reproductive dysfunction, among others. Although the gonads have long been considered the major reproductive target of EDCs, recent data underline their impact on the developing brain, especially on the Gonadotropin Releasing Hormone (GnRH) neuronal network. The GnRH network, situated in the hypothalamic region of the brain, orchestrates the timing of sexual maturation and reproductive function. The programming of these functions is finely tuned by sex steroids and is highly sensitive to early environmental factors. It is now well established that developmental exposure to EDCs can lead to long-lasting health consequences. Early EDC exposure has been associated with advanced or delayed pubertal onset in both boys and girls and accounts as one of the main factors explaining the trend toward advanced pubertal timing in the last centuries. Additionally, developmental EDC exposure is able to alter later in life, inducing ovarian and reproductive function as polycystic ovarian syndrome (PCOS) or early menopause. One of the first unfortunate evidence of the adverse effects of EDCs comes from the observations of patients exposed to Diethylstilbe-

strol (DES), a drug prescribed to pregnant women from 1940 to 1971 to allegedly prevent miscarriage. Not only DES was not effective against miscarriage, but fetal exposure to this potent estrogenic compound produced a rare vaginal tumor and reproductive malformations that could even impact the next generation.

In recent years, there is an increased concern on the potential consequences of EDCs across generations. The proposed state that factors related to one's lifestyle or experiencing dramatic historical events could have consequences in future generations, increasing the prevalence of disease. For instance, during the second World War, a certain part of the population in the Netherlands suffered from a famine. The offspring exposed to the famine during gestation suffered from diminished glucose tolerance and displayed increased rates of obesity. Interestingly, their grandchildren, which were not directly exposed to the famine, were found to have increased neonatal adiposity. The study of such phenomenon caused by EDC exposure in humans is a real challenge - if not infeasible- but rodent studies have demonstrated evidence in favor of this hypothesis. Developmental exposure to EDCs was found to alter pubertal timing, stress responsiveness and anxiety-like behaviors three generations after the exposure. It is important to note that the third generation is not directly exposed to the compound, suggesting that the effects are transmitted through epigenetic alterations of the germline. How developmental exposure to EDCs can reach and disrupt the germline and, in turn, affect the differentiation and functioning of specific somatic cell subpopulations of the central nervous system is definitely still unknown. However, the cumulative data on the topic raise concerns about the legacy we give to future generations and urges to take a more appropriate risk assessment of these compounds.

Regulatory agencies such as the EPA and the EFSA are in charge of regulating and evaluating the risk assessment of these chemicals. The EDC-associated hazard is agreed by the establishment of a threshold (i.e. NOAEL), under which there is no predicted adverse effects for each chemical. However, humans are not exposed to one single chemical but complex mixtures of compounds. The diversity of chemicals that different subpopulations and social sectors are exposed to, makes it difficult to predict the potential adverse outcomes of EDCs. Additive, antagonistic and synergistic effects have been observed after exposure to EDC mix-

tures. Additionally, EDCs can induce effects in a nonlinear and non-monotonic dose response fashion, which implies that exposure to extremely low doses can also produce adverse effects. Our laboratory has demonstrated adverse effects of the plasticizer BPA at doses in the nanogram range in female sexual maturation, producing opposite effect to those found at high doses. Altogether, these arguments make the current regulatory toxicology fail to encompass real world situations. In this PhD dissertation, we have two aims: (1) to determine the effect of a single compound, the plasticizer BPA, on the reproductive axis after exposure at two different windows of exposure: early neonatal or adulthood. Two doses have been used, a low dose representing the human exposure range and a high contrasting dose. (2) to study the effects of a mixture of 13 common EDCs at low doses in the human exposure range on sexual maturation and maternal behavior accross generations. The study of transcriptional and epigenetic mechanisms related to the reproductive and behavioral phenotypes found in our studies were focused on the GnRH network throughout development. Our original approach identifies the cellular, transcriptional and epigenetic mechanistic and phenotypical consequences of developmental exposure to a mixture of EDCs up to four generations after exposure. These results are extremely important from a conceptual as a regulatory point of view and urges to further evaluate the effects of EDCs across generations in human populations.

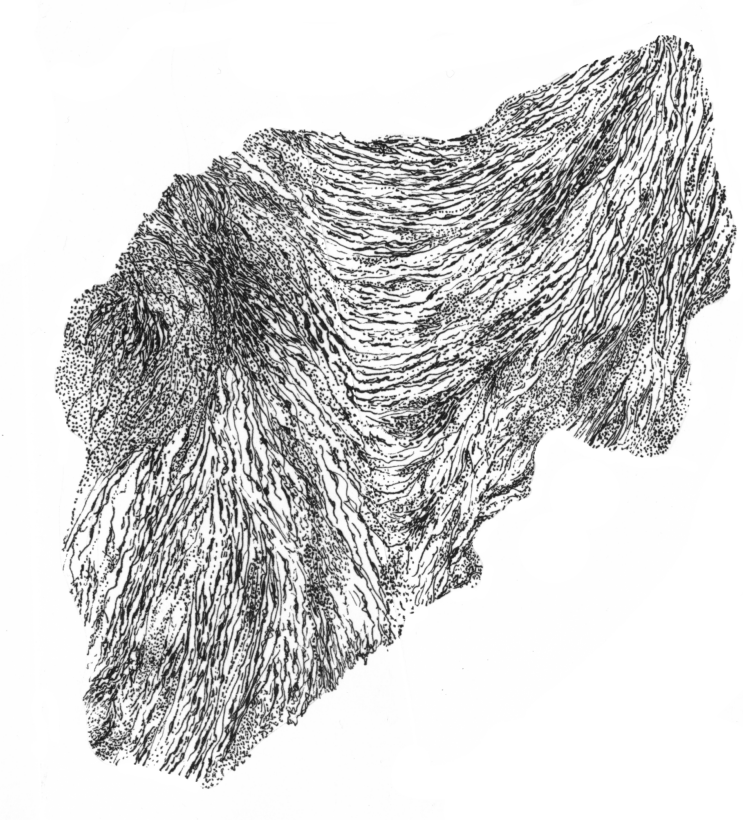
List of abbreviations

3-BC 3-benzylidene camphor
4-MBC 4-methylbenzylidene camphor
4-NP 4-nonylphenol
5hmC 5-hydroxymethylcytosine
5-mC 5-methyl cytosine
ACM acetaminophen / paracetamol
ADI acceptable daily intake
AgRP agouti-related protein
AhR aryl hydrocarbon receptor
AMPA α—amino-3-hydroxy—5—methyl—4—isoxazole propionic acid
AMY amygdala
ARC arcuate nucleus
ATZ atrazine
AVPV anteroventral periventricular
Bax BCL2-associated X protein
BBB blood brain barrier
Bcl2 B cell lymphoma 2
Bdnf brain-derived neurotrophic factor
bFGF basic fibroblast growth factor
Bmal1 aryl hydrocarbon receptor nuclear translocator-like 1
Bmd2 bone mineral density 2
BNST bed nucleus of the stria terminalis
BNSTv ventral bed nucleus of the stria terminalis
BP butylparaben
BPA bisphenol A
BPF bisphenol F
BPS bisphenol S
CART cocaine- and amphetamine-regulated transcript
CeA central amygdala
Clock clock circadian regulator
COU coumestans
CPP central precocious puberty
CTX cortex
DBP dibutyl phthalate
DDT dichlorodiphenyltrichloroethane
DEET n,n-diethyl-metatoluamide
DEHP di(2-ethylhexyl) phtalate
DES diethylstilbestrol
DIBP diisobutyl phthalate
DNMT DNA methyltransferase
DoHaD developmental origin of health and disease
DPN diarylpropionitrile
Eap1 Enhanced at Puberty 1
EASs endocrine active substances
EDCs Endocrine disrupting chemicals
EE3 ethinylestriol
EFSA European Food Safety Authority
EP2 prostaglandin E_2 receptor
EPA U.S. Environmental Protection Agency
EQ equol
ERα estrogen receptor alpha
ERβ estrogen receptor beta
ERBB family of receptor tyrosine kinases
Esr1 estrogen receptor 1
Esr2 estrogen receptor 2
FAD flavin adenine dinucleotide
FSH follicle stimulating hormone
GABA gamma-Aminobutyric acid
GAD glutamate decarboxylase
Gad2 glutamic acid decarboxylase 2
Gat2 glutamic acid transporter 2
GEN genistein
GnRH gonadotropin releasing hormone
Grin2d glutamate Ionotropic Receptor NMDA Type Subunit 2D
GWAS genome-wide association study
HATs histone acetyl transferases
HDACs histone deacetylases
HDMs histone demethylases
HIP hippocampus
HMTs histone methyl transferases
HPA hypothalamic pituitary adrenal axis
IGF-1 insulin growth factor 1
IQR interquartile
Kiss1 kisspeptin 1
KOR K-opioid receptor
LG licking and grooming
LH luteinizing hormone
lncRNA long non-coding RNA
LOAEL lowest observed adverse effect level
MAT methionine adenosyltransferase
Mbd2 methyl binding domain 2

MBH mediobasal hypothalamus
MeA median amygdala
Meg3 maternally expressed gene 3
MeP methylparaben
miRNA micro RNA
Mkrn3 makorin RING-finger protein 3
mPoA median preoptic area
MTX methoxychlor
NA nucleus accumbens
ncRNA non-coding RNA
NKB neurokinin B
NMDA N-methyl-d-aspartate
NMDRC nonmonotonic dose-response curve
NO nitric oxide
NOAEL no observable adverse effect level
NP nonylphenol
NPY neuropeptide Y
OB Olfactory bulbe
OECD Organisation for Economic Cooperation and Development
OMC octyl methoxycinnamate
OP octylphenol
OVLT organum vasculosum of the lamina terminalis
PACAP pituitary adenylate cyclase activating polypeptide
PAG periaqueductal gray area
PBDEs polybrominated diphenyl ethers
PcG polycomb group of proteins
PCOS polycystic ovary syndrome
Per1 period circadian protein homolog 1
Per2 period circadian protein homolog 2
PFC prefrontal cortex
PFOA perfluorooctanoic acid
PFOS perfluorooctanesulfonic acid
PGCs primordial germ cells
PGE_2 prostaglandin E_2
piRNA piwi-interacting RNA
PND postnatal day
Pnx phoenixin
PoA preoptic area
POMC pro-opiomelanocortin
PP propylphenol
PPT propylpyrazole triol
PrP propylparaben
PTM post-translational modification
qPCR quantivative polymerase chain reaction
SAM S-adenosyl methionine
SCN suprachiasmatic nucleus
SEMA3A semaphorin 3A
SEMA7A semaphorin 7A
SIRT1 Sirtuin 1
SN substantia nigra
sncRNA small non-coding RNA
STAR steroidogenic acute regulatory protein
SWI/SNF switching defective/sucrose non/fermenting
TBBPA tetrabromobisphenol A
TBT tributyltin
TCDD 2,3,7,8-tetrachlorodibenzo-p-dioxin
TCS triclosan
TET ten-eleven translocation methylcytosine dicoxygenases
TGFα transforming growth factor alpha
TGFβ transforming growth factor beta
Trp53 transformation-related protein 53
TrxG trithorax group of proteins
TSS transcription start site
Ttf1 thyroid transcription factor 1
VIN Vinclozolin
VMH ventromedial hypothalamus
VTA ventral tegmental area
WoE window of exposure
ZEA zearalenone

CONTENTS

INTRODUCTION



1 The hypothalamic control of sexual maturation

Contents

In this chapter we will discuss the major excitatory and inhibitory transsynaptic and glial factors controlling GnRH secretion among a synchronized hypothalamic network finely control the frequency and amplitude of GnRH pulses. We will also summarize the epigenetic control of the transcriptional switch that leads to activation of the GnRH system at puberty.

1.1 The HPG axis: GnRH neurons

Sexual maturation, eventually leading to the acquisition of reproductive function, is characterized by the activation of an intricate network of neurons and glia cells in the hypothalamic region of the central nervous system (Fig 1.1). Such hypothalamic network orchestrate the activity of the gonadotropin releasing hormone (GnRH) neurons, which control and modulate the peripheral synthesis and release of steroid hormones by the gonads (Plant, 2015). GnRH neuron cell bodies are situated mainly in the median preoptic area (mPoA), while their axonal terminals, secrete pulsatile GnRH into the hypophysial portal system. GnRH travels to the pituitary gland where it stimulates the secretion of gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH). Gonadotropins are released into the peripheral circulation and reach the gonads where they stimulate the synthesis of steroid hormones as well as gametogenesis. An estrogen (E2) negative feedback occurs in both males and females, when E2 increases in peripheral circulation and decrease the central release of GnRH. In postpubertal females, an estrogen positive feedback system occurs in order to induce the preovulatory LH surge leading to ovulation. Because of the central role played by GnRH neurons in pubertal onset and ovulation, it is of interest to discuss the development, morphology, localization and function of these neurons.

GnRH neurons originate from progenitors in the olfactive placode. They appear around embryonic day 13 in rats (Schwanzel-Fukuda and Pfaff, 1989), 32 in monkeys (Ronnekleiv and Resko, 1990) and during the fifth week of gestation in humans (Cummings and Brunjes, 1995). In the rat, GnRH neurons migrate through the anterior brain to their final destination in the hypothalamus at embryonic day 17 and 18 (Schwanzel-Fukuda and Pfaff, 1989; Yoshida et al., 1995). This unique migration depends on fibroblast growth factor and prokineticin signaling, as well as Anosmin-1 interaction with heparin sulfate glycosominoglycan proteins within the extracellular matrix (Dode et al., 2006; Franco et al., 1991; Chung et al., 2016; Malone et al., 2019). Alterations in any of this finely tuned mechanisms results in Kallmann Syndrome, characterized by the absence of puberty that can be accompanied with anosmia, synkinesia, dental agenesis, hearing loss or digital bony abnormalities (Dodé and Hardelin, 2009). While most GnRH

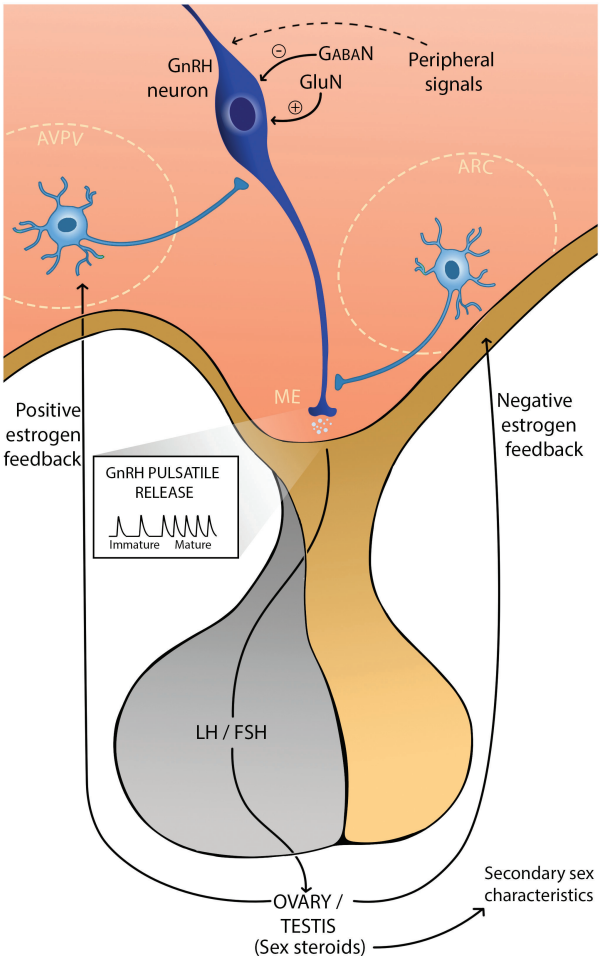


Figure 1.1. Hypothalamic pituitary gonadal (HPG) axis. GnRH neurons integrate peripheral, neuronal (ex. GABAergic and glutamatergic neurons), hormonal and glial signals to trigger gonadotropin and sex steroid release. Sex steroids released by the gonads maintain gonadotropin levels by the action of a negative feedback. Thus, a positive feedback triggers ovulation in females.

neurons are diffusely located in the mPoA and the median septum in the rat, a small number is found scattered within the basal forebrain (Barry, Dubois, and Poulain, 1973; Silverman, Silverman, and Gibson, 1989). However, GnRH neurons are relatively few in number, approximately 1,300 in rats, 800 in mice and around 1,000 to 1,500 in humans (Millar, 2005; Balasubramanian et al., 2010; Wray and Hoffman, 1986; Jasoni, Porteous, and Herbison, 2009). GnRH neuron axons mainly extend their projections through the tuberoinfundibular tract to the median eminence, at the floor of the third ventricle, immediately caudal to the optic chiasm where they gain access to the hypophyseal portal system (Plant, 2015). Studies have identified GnRH afferences in the cortex, the periaqueductal grey nucleus and the amygdala (Boehm, Zou, and Buck, 2005; Buma, 1989; Witkin, Paden, and Silverman, 1982; Liposits and Setalo, 1980; Sakuma and Pfaff, 1979). Immunohistochemical studies of the GnRH neuron morphology have shown that they are bipolar and fusiform-shaped with both dorsal and ventral dendritic extensions. During postnatal development, GnRH neurons changes substantially their morphology by doubling their dendritic tree and spine density (Cottrell et al., 2006). It is now well established that such extensions have a double role, acting simultaneously as axons and dendrites, which lead to the name of "dendrons" (Herde et al., 2013). This structure allows GnRH projections to receive and integrate synaptic inputs at the same time while initiating and propagating action potentials, providing a greater dynamic control of GnRH secretion at the level of the presynaptic terminal in the median eminence and their soma.

Throughout development, both frequency and amplitude of GnRH secretion play an essential role in the onset of the reproductive function and evolve throughout development (Knobil, 1980). A pulsatile pattern is essential, as constant infusion of exogenous GnRH in rhesus monkeys with hypothalamic lesions are not able to sustain gonadotropin secretion, while pulsatile infusion does (Belchetz et al., 1978). GnRH neurons may possess an intrinsic pacemaker mechanism as *in vitro* culture of GnRH neurons isolated from the olfactive placode are able to secrete GnRH in a pulsatile manner (Funabashi et al., 2000; Terasawa et al., 1999; Duittoz and Batailler, 2000). Moreover, electrophysiological studies (reviewed in Moenter, 2010) using brain slices or culture of embryonic GnRH neurons provided evidence of spontaneous bursting activity that persists after block-

ing GABAergic and glutamatergic signals (Kuehl-Kovarik et al., 2002; Abe and Terasawa, 2005; Nunemaker, DeFazio, and Moenter, 2002; Sim, Skynner, and Herbison, 2001; Spergel et al., 1999; Suter et al., 2000). However, GnRH pulsatility has also been described to originate in extrinsic factors. Female rat hypothalamic explants missing the preoptic area where GnRH soma are located show GnRH pulsatility (Purnelle et al., 1997) *ex vivo*. Experiments from our laboratory and others have shown that neurons crucial for GnRH pulsatility are located in the arcuate nucleus (ARC) of the hypothalamus (Halasz and Gorski, 1967; Soper and Weick, 1980; Blake and Sawyer, 1974). The electrical activity of ARC was found to be correlated with GnRH secretion in both rhesus monkeys (Wilson et al., 1984; O'Byrne and Knobil, 1993) and rats (Kimura et al., 1991). It is now known that the principal factor accounting for GnRH pulsatility within the ARC are the KNDy neurons, co-expressing kisspeptin, neurokinin B and dynorphin (Seminara et al., 2003; Han et al., 2015). Moreover, an endogenous clock in the suprachiasmatic nucleus (SCN) also affects GnRH pulsatility (Patel et al., 2017). The role of kisspeptin and circadian clock genes in the control of GnRH pulsatile secretion will be discussed further in the next chapters. Globally, while GnRH neurons have the capability of generating synchronous and spontaneous bursting, GnRH frequency seems to be regulated by integrated signals derived from neural, hormonal and glial inputs.

1.2 Developmental changes in GnRH secretion

The GnRH network follows a complex developmental process, including cellular, transcriptional and epigenetic modifications that reorganize the neuroendocrine and glial network controlling reproduction. From birth to adulthood, we can identify four stages where GnRH frequency and amplitude change ultimately leading to the activation of the neuroendocrine system at puberty (Fig. 1.2).

During early neonatal life, GnRH frequency increases rapidly after birth, as consequence of the disappearance of the negative feedback caused by the decrease in circulating placental steroids (Lanciotti et al., 2018; Plant, 2015). The physiological meaning of this developmental stage called minipuberty remains unclear but it has been associated with gonadal growth and increased testosterone levels (Boas et al., 2006; Kiviranta et al., 2016). Minipuberty, which takes place around

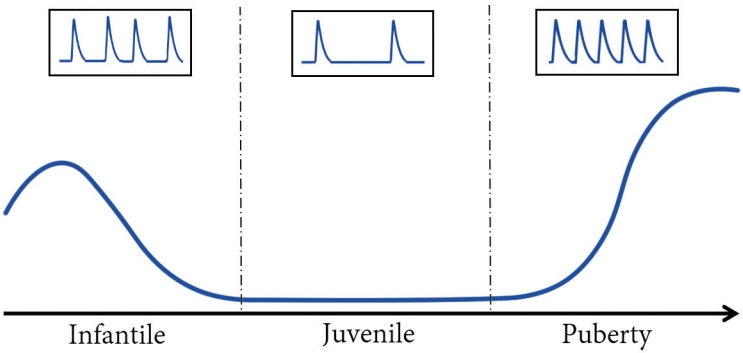


Figure 1.2. Representation of changes in GnRH pulsatile secretion throughout development, from infantile to pubertal onset in female rats. GnRH frequency at every stage of development is indicated in insets.

day 12 in rat and mice, is followed by a quiescent juvenile period, where GnRH frequency and amplitude remain low. This low GnRH activity is explained by potent trans-synaptic inhibition at the level of the GnRH neuronal network (Ramirez and McCann, 1963)and sensibility to estrogen negative feedback (Kulin, Grumbach, and Kaplan, 1969). Thereafter, starting from day 12, GnRH secretion progressively rises, especially during the afternoon, in both males and females. This reawakening of the GnRH neuronal network is caused by the decrease in trans-synaptic inhibition and the increase in expression of excitatory factors within the hypothalamus (Ojeda and Skinner, 2006; Selmanoff, Goldman, and Ginsburg, 1977). During this prepubertal period LH pulses are detected every 2-3h in mice (Coquelin and Desjardins, 1982) and and decrease to 90 minutes per pulse at birth to 40 minutes per pulse at the age of 25 days in the rat (Yamanaka et al., 1999). In our laboratory, rat hypothalamic explant incubation *Ex vivo* from day 5 to 25 has shown increased GnRH frequency throughout development (Bourguignon and Franchimont, 1984b). In the rat, the first endocrine manifestation of the onset of puberty is a circadian increase in gonadotropin during the prepubertal period around day 28-30 (Boyar et al., 1972; Urbanski and Ojeda, 1987).

During the pubertal stage, while GnRH levels remains high in males, females display periodic changes starting with the first estrous cycle. Such variations will be discussed further in the next sections and chapters. In the female rat, the first

visible manifestation of puberty is vaginal opening (around day 32 to 38), followed by estrous cycle (Goldman, Murr, and Cooper, 2007; Ojeda et al., 1976). In humans, the first manifestation of puberty in girls is breast development around 10 years of age followed by the first menarche around 12 to 13 years.

1.3 The GnRH network: factors controlling GnRH secretion

Pulsatile GnRH release depends on excitatory and inhibitory transsynaptic inputs to GnRH neurons or adjacent cells. Neurotransmitters and neuropeptides, in particular GABA, glutamate, Kisspeptins, Neurokinin B, Dynophin and RFamide peptides play a critical role in the regulation of GnRH secretion (Terasawa et al., 2018; Zhang et al., 2009; Bourguignon, Gerard, and Franchimont, 1989). On the other hand, glial cells impose a stimulatory effect to the GnRH neuronal network through secretion of prostaglandins and growth factors (Ojeda and Skinner, 2006; Plant, 2015; Ojeda, Lomniczi, and Sandau, 2010; Prevot et al., 2003). Only factors related to the experiments carried out in the present dissertation will be discussed in the next sections.

1.3.1 GABA / Glutamate

The neurotransmitters gamma-Aminobutyric acid (GABA) and glutamate are the main inhibitory and excitatory components of the central nervous system and two of the major regulators of GnRH secretion (Krnjević, 2004; Watanabe, Fukuda, and Nabekura, 2014).

Glutamate is synthesized from the precursors glutamine, $\alpha-$ketoglutarate and 5-oxoproline (Fig.1.3). Astrocytes release glutamine by recycling glutamate and other factors, that will be used by neurons as precursor to synthesize glutamate and GABA (Cooper, 1987). When glutamate is released into the synaptic cleft it binds to ionotropic and metabotropic receptors. There are three different types of ionotropic receptors named by their selective agonists: N-methyl-d-aspartate (NMDA), $\alpha-$amino-3-hydroxy$-5-$methyl$-4-$isoxazole propionic acid (AMPA) and kainate (Dawson, 1999). Among the NMDA receptors, there are three major subunit families, represented by one or several genes (*Grin1*, *Grin2a-d* and *grin3a*) and are permeable to Na^+. NMDA subunits are differen-

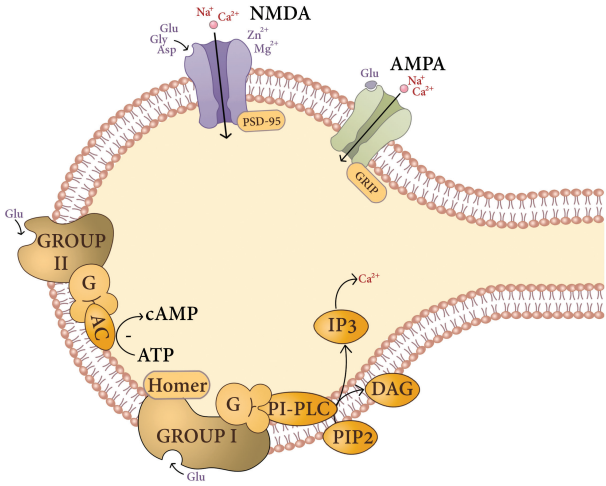


Figure 1.3. Schematic view of the glutamatergic ionotropic and metabotropic receptors.The ionotropic receptors AMPA and NMDA, as well as the metabotropic receptors Group I and Group II are shown. Group I correspond to the *mGlu1* and *mGlu5*, activators of the phospholipase C (PLC) transduction pathwhay and Group II correspond to the activators of the adenylyl cyclase (*mGlu2-4* and *mGlu6-8*).

tially distributed among the central nervous system. While *Grin1* and *Grin3a* are widely distributed within the brain, except for the cerebellum, *Grin2a-d* has a most restrictive pattern of expression in the midbrain and the forebrain. AMPA and kainate receptors have four (*GluR1-4*) and five subunits (*GluR5-7* and *Ka1-2*) respectively and are permeable to K^+ and Ca^{2+} (Dawson, 1999). Among these subtypes, *GluR1-2* are the most widely expressed in the hypothalamus (Van Den Pol et al., 1994). The metabotropic receptors are eight G—protein coupled receptors that can be subdivided based on their transduction mechanisms, either by activating phospholipase C (*mGlu1* and *mGlu5*) or adenylate cyclase (*mGlu2-4* and *mGlu6-8*).

Glutamate plays a crucial role in activating GnRH neurons, in particular at the time of puberty (Clarkson and Herbison, 2006a; Terasawa et al., 1999). While GnRH neurons express all glutamate ionotropic receptors (AMPA, NMDA and kainate), the site of glutamate action within the GnRH neuronal network remains to be completely elucidated (Iremonger et al., 2010). It has been proposed that glutamatergic input into GnRH neurons comes from the anteroventral periven-

tricular (AVPV) region of the hypothalamus, which is known to send projections to GnRH neurons (Simonian, Spratt, and Herbison, 1999; Hutton, Gu, and Simerly, 1998). However, it is known that glutamate ionotropic receptors are found in both the GnRH neuron terminals in the median eminence and in the postsynaptic density, an electron dense structure mainly found on GnRH dendritic spines (Iremonger 2010). The role of metabotropic glutamate receptors remains unclear (Iremonger et al., 2010).

The three types of glutamatergic receptors play differential roles in activating GnRH secretion. In an *in vitro* model using GnRH neurons from mouse, AMPA increased cellular calcium concentration in GnRH neurons by 80% (Constantin, Iremonger, and Herbison, 2013). When stimulating NMDA receptors, the increase in intracellular calcium was only of 10-15% (Constantin, Iremonger, and Herbison, 2013). However, pulsatile GnRH secretion was decreased by an NMDA antagonist but not when blocking AMPA or Kainate receptors in rat hypothalamic explants incubated *ex vivo* (Bourguignon, Gerard, and Franchimont, 1989). This data suggests a major role for NMDA receptors NMDA in the activation of GnRH secretion. Supporting this results, intermittent administration of NMDA in juvenile rhesus monkeys (GAY and PLANT, 1987) and rats (Smyth and Wilkinson, 1994; Urbanski and Ojeda, 1987) induces precocious pubertal onset. In the rat, the response was higher when NMDA was administered around day 25 (Cicero et al., 1988; Bourguignon et al., 1992), period of increased number of glutamate receptors in GnRH neurons. Moreover, administration of the NMDA antagonist MK-801 delayed puberty in the rat (Meijs-Roelofs, Kramer, and Leeuwen, 1991). Expression of glutamatergic receptors in the preoptic area increase throughout development peaking at puberty (Goroll, Arias, and Wuttke, 1993). Overall, this suggests that there are developmental changes in the sensitivity of GnRH neurons to glutamatergic input and that glutamate plays a major role in pubertal onset through the activation of NMDA receptor in GnRH neurons.

GABA is synthesized from glutamate via the enzyme glutamate decarboxylase (GAD) and the cofactor pyridoxal phosphate (the active form of vitamin B6). GABA is then stocked by the vGAT transporter into synaptic vesicles and released by exocytosis into the synaptic cleft (Modi, Prentice, and Wu, 2015). There are two isoforms of the GAD enzyme but only the GAD65 (Gad2) is in-

volved in neurotransmission as is located within the synaptic terminals. Two different post-synaptic receptors can be activated by GABA, the receptor $GABA_A$ and $GABA_B$. The $GABA_A$ ionotropic receptors, composed of five subunits, opens anion-selective channels predominantly conducting chloride ions (Cooper, 1987). The $GABA_B$ metabotropic receptors are G protein coupled receptors found in the pre- and postsynaptic sites.

As one of the major regulators of GnRH secretion, developmental changes in GABAergic tonus play a crucial role in pubertal onset. In children suffering from seizures and in mice, administration of the GABAergic agonist valproic acid delays pubertal timing (Lundberg et al., 1986). Precocious puberty was observed in an 11 month-old girl with nonketotic hyperglycinemia. Due to high concentrations of glycine acting on NMDA receptors as a coagonist of glutamate. Regression of pubertal development during anticonvulsive treatment with GABA agonists suggested that the stimulatory effects of glycine could be overcome by GABA receptor-mediated inhibition. (Bourguignon et al., 1997). The same GABA agonists were able to suppress the developmental increase in the frequency of pulsatile GnRH secretion. These studies suggest a clear inhibitory role of GABA in GnRH secretion and puberty. However, while the neurotransmitter GABA is generally inhibitor, it stimulates GnRH neurons when directly acting at their level (reviewed in Watanabe, Fukuda, and Nabekura, 2014). An added level of complexity arised from the observation that GABA can also inhibit GnRH secretion indirectly via effects exerted on neurons connected to the GnRH neuronal network (Ojeda and Skinner, 2006; Terasawa, 2006). Although most GnRH neurons are excited by GABA, its effect is likely determined by the balance between $GABA_A$ and $GABA_B$ receptors in their soma and dendrites (Watanabe, Fukuda, and Nabekura, 2014). GABA plays a role at different developmental stages of the hypothalamic-pituitary-gonadal axis. First, it is involved in the regulation of GnRH neuron migration (Heger et al., 2003; Lee et al., 2008). Later on, a decrease in GABAergic tone is crucial for the reactivation of the GnRH machinery at puberty (Han, Abraham, and Herbison, 2002; Parent, Matagne, and Bourguignon, 2005) and appears to also control the switch in E2 feedback action in adult females (Farkas et al., 2018). The inhibitory effect of GABA on GnRH neurons is especially predominant during postnatal day 5 to 15 in the female

rat (Parent, Matagne, and Bourguignon, 2005) and decreases throughout development (Bourguignon et al., 1997), concomitantly with a decrease in GABAergic levels in the preoptic area (Goroll, Arias, and Wuttke, 1993). The $GABA_{A\delta_2}$ subunit is known to be highly expressed before birth and decreases gradually until adulthood. $GABA_{A\delta_1}$ subunit follows the opposite pattern, increasing during development (Terasawa, 2006). The profile of other $GABA_A$ subunits follows similar changes (Sim et al., 2000). In juvenile rhesus monkeys, it was found that reducing GABA synthesis by interference with both isoforms of GAD in GnRH release (Terasawa et al., 1999; Mitsushima et al., 1996). Overall, this cumulative knowledge points at GABA as a major player in the regulation of GnRH release at puberty.

1.3.2 Kisspeptin

Kisspetins has been discovered recently as one of the principal factors controlling hypothalamic pulsatile GnRH secretion, the onset of puberty and ovulation. Kisspeptins are a group of related peptides derived from a common 145 amino acid precursor encoded by the *Kiss1* gene (Kotani et al., 2001; West et al., 1998). The *Kiss1* gene consists of 3 exons where only a partial segment of the second and thrid are translated. Cleavage of the 145 amino acid precursor produces 54, 14, 13 and 10 amino acid active kisspeptins that have the same affinity for GPR54 receptor(Kotani et al., 2001; West et al., 1998; Li, Yu, and Liu, 2009). In the rat hypothalamus, two different transcriptional start sites (TSS) have been identified (Mueller et al., 2011; Castellano et al., 2014). TSS2 producing a longer transcript than TSS1. However, only the expression of TSS1-derived transcrips change at puberty (Castellano et al., 2014). These differences were found to be related to the presence of binding sites to transcription factors and estrogen-responsive elements found proximal to the TSS1 promoter, but not TSS2. Kiss1 promoter regulatory region is located upstream of the TSS. This region contains a site at -188 to -87 and binding sites for specific protein 1 (*Sp1*) and 3 (*Sp3*), which mediates the regulation of *Kiss1* expression by estradiol (Li et al., 2007).

While first identified as metastasis suppressors, the crutial role of kisspeptin in the regulation of GnRH secretion was described in (Seminara et al., 2003; Roux et al., 2003). In humans, mutations of the Kisspeptin G protein coupled receptor

Gpr54 are associated with hypothalamic hypogonadism (Seminara et al., 2003; Roux et al., 2003). Thereafter, kisspeptins has been shown as a major activator of GnRH secretion (Han et al., 2005; Nazian, 2006). Using *in vitro* incubation of mouse hypothalamic section, GnRH neuron activity was found to be increased in the presence of kisspeptin at low concentrations (Liu, Lee, and Herbison, 2008; Liu and Herbison, 2011). Kisspeptin also stimulates GnRH secretion from mediobasal hypothalamic explants (MBH) explants *ex vivo* (Uenoyama et al., 2011; Tassigny and Colledge, 2010). As the MBH does not contain GnRH cell bodies, these studies suggest that kisspeptin triggers GnRH release by a direct action on GnRH nerve terminals. Moreover, another recent study using electrophysiological recording has shown that kisspeptin neurons were also able to specifically activate the GnRH dendritic trees that have afferences to the organum vasculosum of the lamina terminalis (OVLT) (Herde et al., 2011). GnRH dendrites in this regions are involved in the LH preovulatory surge and were found to be outside the blood brain barrier (BBB) (Herde et al., 2011). Kisspeptin activation of GnRH neurons not only happens through direct action but also via transsynaptic connections through GABAergic and nitric oxide (NO) neurons (Hanchate et al., 2012; Pielecka-Fortuna, Chu, and Moenter, 2008).

In mammals, kisspeptin-containing neurons are found in two hypothalamic regions: the rostral hypothalamic region AVPV of the hypothalamus and the caudal region of the arcuate nucleus (ARC) (Tassigny and Colledge, 2010; Kauffman et al., 2007a). The two kisspeptin populations play a different role in the control of the reproductive function. AVPV kisspeptin neurons send projections directly to the GnRH soma (Clarkson and Herbison, 2011; Clarkson and Herbison, 2006b), which expresses *Gpr54* receptors (Irwig et al., 2004; Messager et al., 2005). AVPV kisspeptin neurons express sex steroid receptors and respond to gonadal steroids (Smith et al., 2005b) as well as to circadian cues (Kriegsfeld, 2013). During proestrus, estrogens stimulate kisspeptin synthesis and release from AVPV kisspeptin neurons and induce the preovulatory LH surge leading to ovulation (Clarkson and Herbison, 2006b; Smith et al., 2011; Adachi et al., 2007).

The kisspeptin neuron population in the ARC, so called KNDy neurons, because they co-express the peptides neurokinin B (NKB) and dynorphin, are now known to be a major component of the GnRH pulse generator (Navarro et al.,

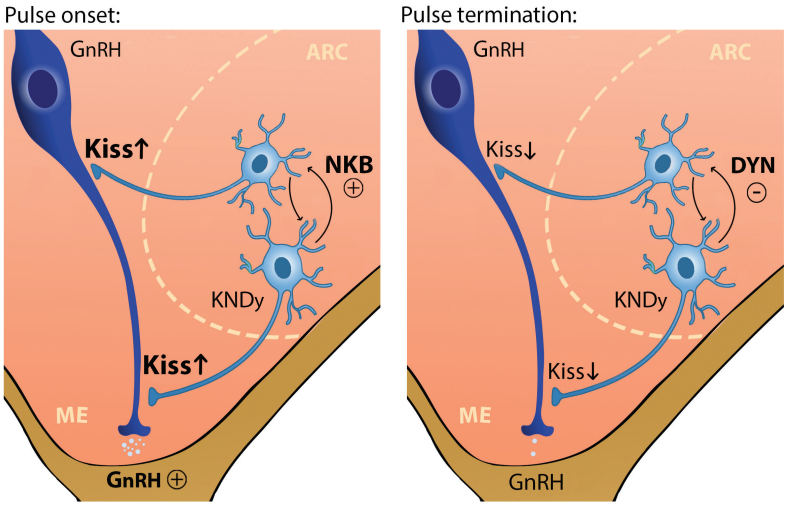


Figure 1.4. Model of the control of the GnRH pulse generator drive by KNDy neurons. In this model (left), NKB secretion within the interconnected KNDy neurons leads to the synchronious release of kisspeptin into GnRH that triggers the GnRH pulse. GnRH pulse is initiated by the direct action of kisspeptin into GnRH neuron and distal dendrites expressing GPR54. Thereafter (right), GnRH pulse is suppressed by the inhibitory action of dynorphin secreted by KNDy neurons, which decreases NKB release. Dynorphin can repress GnRH release by direct action on GnRH neurons. Dashed circles represent the ARC.

2009a) (Fig.1.4). In KNDy neurons, gonadal estrogens suppress kisspeptin expression via ERα/*Esr1* (Smith et al., 2005b). Selective deletion of Esr1 in KNDy neurons leads to advanced puberty and elevated LH levels (Kauffman et al., 2007a; Kauffman et al., 2009). KNDy neurons connect directly to the soma and distal dendrites of GnRH neurons (Herde et al., 2013). The use of KNDy neurons knockdown models, lesions in the ARC or targeted optogenetic inhibition, have unequivocally proven that this population plays a role in estrous cyclicity and the regulation of GnRH frequency and amplitude (Beale et al., 2014; Hu et al., 2015; Mittelman-Smith et al., 2012; Clarkson et al., 2017). KNDy neurons form a synchronized network sending rhythmic signals to GnRH secretion, allowing for its pulsatile secretion. As KNDy neurons also express the tachykinin NKB and dynorphin receptors, kisspeptin neurons within the network can respond to the kisspeptin secretion of other members of the network(Navarro et al., 2011;

Gottsch et al., 2011; Croft, Boehm, and Herbison, 2013; Goodman et al., 2007). In rodents, depolarization of KNDy neurons is followed by activation of the NKB receptors NK3R (encoded by the gene *Tac3r*) and Neurkinin 1 (NK1) found in KNDy neurons, stimulating kisspeptin secretion (Croft, Boehm, and Herbison, 2013). In contrast, dynorphin secreted within the network inhibits kisspeptin release (Moore et al., 2018). This model suggests that GnRH pulses are generated by the stimulatory action of kisspeptin when NKB is secreted within the KNDy network and that dynorphin serves as a signal to terminate each pulse(Navarro et al., 2009a). Supporting this model, NKB receptors are not found in GnRH neurons neither in sheep or rodents, suggesting that its stimulatory role remains within the KNDy neurons (Burke et al., 2006; Goodman et al., 2007). However, dynorphin can directly inhibit GnRH neurons that GnRH neurons express the dynorphin K-opioid receptor (KOR) (Weems et al., 2016).

The distribution of kisspeptin neurons varies among species and genders. In rodents, AVPV kisspeptin neurons are sexually dimorphic, females having a greater number of kisspeptin neurons as compared to males. Gonadectomized juvenile male rats display an increase in Kiss1 mRNA expression in the AVPV (Kauffman et al., 2007a; Adachi et al., 2007), while sex steroids administration reverses this effect. In contrast to the AVPV, the distribution and number of KNDy ARC neurons is not sexually different in rodents (Clarkson and Herbison, 2006a; Kauffman et al., 2007a).

Puberty requires the proper interaction of kisspeptin with GnRH neurons as illustrated by subjects with inactivating mutations of the *Gpr54* gene (Roux et al., 2003; Seminara et al., 2003) who do not undergo spontaneous puberty for most of them. During the juvenile period, the *Kiss1* gene is repressed leading to low GnRH pulses. Expression and secretion of kisspeptin as well as the number of synaptic appositions between kisspeptin and GnRH neurons increase during the prepubertal period (Shahab et al., 2005; Desroziers et al., 2012; Navarro et al., 2004; Tena-Sempere, 2010). Concomitantly, GnRH neuron sensibility to kisspeptin input increases at the same time (Han et al., 2005). The activation of kisspeptin expression happens during the prepubertal period in both kisspeptin neuron populations of the AVPV and ARC but not at the same pace. ARC KNDy neurons are activated first as they are required for the increase in GnRH frequency leading to

pubertal onset (Pineda et al., 2010). Once puberty is initiated, AVPV kisspeptin neurons are activated to trigger the LH preovulatory surge in response to the estrogen positiv feedback (Adachi et al., 2007). The activation of kisspeptin neurons leading to puberty has been recently shown to involve changes in chromatin state of the *Kiss1* gene promoter region, which allows the transcriptional switch from repression to activation (Toro et al., 2018; Lomniczi et al., 2013b). The epigenetic mechanism involved in the female control of puberty will be discussed further in section 2.2.

1.3.3 Oxytocin

Oxytocin is a mammallian neurohormone known to play a role in lactation, affiliative and parental behaviors and female sexual maturation (Blanks and Thornton, 2003; Mogi et al., 2014; Insel and Young, 2000; Mitre et al., 2017; Parent et al., 2005).Gonadal steroids, especially E2, regulate oxytocin activity in the hypothalamus (Tribollet et al., 1990; McCarthy et al., 1996; Kloet et al., 1986). Oxytocin is synthesized by magnocellular neurosecretory cells in the supraoptic and paraventricular nucleus of the hypothalamus, sending most of its projections to the posterior pituitary (also called neurohypophysis) (Brown et al., 2013).

The role for oxytocin in the onset of sexual maturation has been described in recent years. During the prepubertal period there is an increase in the hypothalamic content of oxytocin (Jackson and George, 1980). Chronic administration of an oxytocin antagonist during the prepubertal period produces a decrease in GnRH pulsatility and delays vaginal opening (Parent et al., 2005). These results confirm previous studies showing a facilitatory role of oxytocin in the control of GnRH secretion (Selvage and Johnston, 2001; Rettori et al., 1997). Direct action of oxytocin on GnRH neurons has not been well established as it seems that only a few number of GnRH neurons contain oxytocin receptor. Oxytocin effects during the prepubertal period appears to be mediated by a glia-to-neuron pathway (Parent et al., 2008). Astrocytes in the hypothalamus produce and secrete prostaglandin E_2 (PGE_2) in response to oxytocin, ultimately increasing GnRH secretion, as was previously demonstrated (Ojeda, Naor, and Negro-Vilar, 1979).

1.3.4 Glial factors

A subset of hypothalamic glial cells play a critical role in pubertal onset (Garcia-Segura, Lorenz, and DonCarlos, 2008) and the control of GnRH secretion throughout the estrous cycle (Prevot et al., 2010). Glial cells such as astrocytes and tanycytes release growth factors (TGFα and TGFβ) and prostaglandins (PGE_2) that directly stimulate GnRH release from GnRH neurons (Ojeda, Naor, and Negro-Vilar, 1979; Clasadonte et al., 2011) or indirectly through the control of neurotransmitter release (Glanowska and Moenter, 2011) (Fig.1.5).

At the time of puberty, hypothalamic astrocytes increase secretion of PGE_2, that in turn stimulate GnRH release (Ojeda, Naor, and Negro-Vilar, 1979; Prevot et al., 2003; Clasadonte et al., 2011). Prostaglandin E2 receptor (EP2) in GnRH neurons, respond to the action of PGE_2, whereas ERBB respond to TGFα (Clasadonte et al., 2011). Moreover, PGE_2 can also indirectly stimulate GnRH release through increasing glumatate or inhibiting GABAergic input onto GnRH neurons (Glanowska and Moenter, 2011). The increase in prostaglandins is accompanied by an increase in the hypothalamic expression of TGFα and its associate receptor ERBB1 (Ma et al., 1992; Ma et al., 1999). Furthermore, TGFα secreted by hypothalamic astrocytes binds to astrocytic ERBB1 and ERBB2 receptors (Ma et al., 1992; Ma et al., 1999), causing receptor dimerization and further stimulation of PGE_2 synthesis (Ojeda and Skinner, 2006; Prevot et al., 2007; Prevot et al., 2003). Inactivation of ERBB1 or TGFα induces a delay in puberty (Prevot et al., 2005). The role of TGFβ in puberty is not clear. TGFβ increases GnRH mRNA in the hypothalamus but does not increase GnRH release *in vivo* (Bouret et al., 2004; Melcangi et al., 1995; Gonzalez-Manchon et al., 1991). In a recent *in vitro* study using the premigratory GnRH-(1–5) cell line, GnRH migration was shown to require TGFβ expression (Larco et al., 2018). Other glial factors such as insulin growth factor 1 (IGF-1) and the basic fibroblast growth factor (bFGF) stimulate GnRH release and are involved in pubertal onset (Voigt et al., 1996; Tsai, Werner, and Weiner, 1995; Tata et al., 2012; Hiney et al., 1996; Hiney, Ojeda, and Dees, 1991). GnRH neurons contain receptors for these factors, specifically the bFGF and IGF1 receptor, respectively (Olson et al., 1995; Tata et al., 2012). Inactivation of FGFR1 delays pubertal onset as IGF-1 was shown to stimulate GnRH secretion by using MBH explants *ex vivo* (Tata et al., 2012).

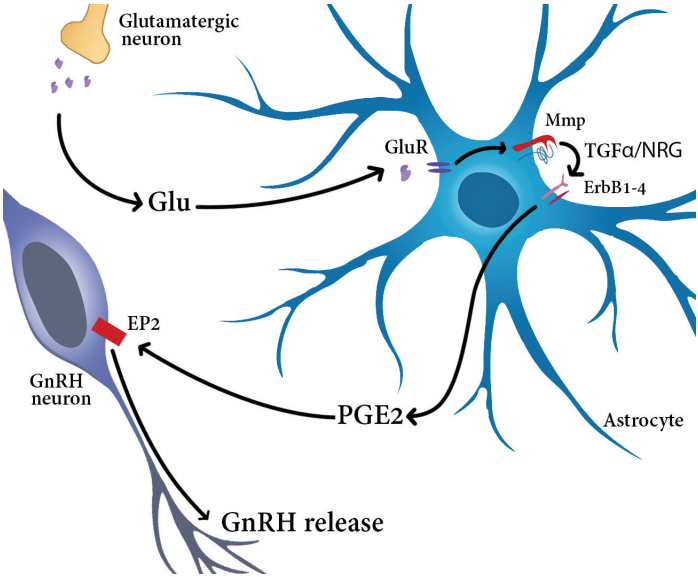


Figure 1.5. Role of hypothalamic astrocytes to stimulate GnRH secretion through the action of prostaglanin PGE_2. PGE_2 release is activated through the activation of the glutamatergic receptors GluR or through the dimerisation of the receptors ERBB1 and ERBB2 caused by TGFα. PGE_2 activates EP2 receptors at GnRH neurons stimulating its release.

Glial cells also regulate GnRH secretion at the level of the GnRH nerve terminals. Tanycytes are specialized ependymoglial cells found in the inner layer of the third ventricle, extending to the median eminence, where GnRH fibers and nerve terminals are found (Prevot et al., 2010). Tanycytes cover GnRH terminals, tightly regulate GnRH release into the vascular endothelial cells in response to estrogen feedback (Seranno et al., 2010). This mechanism is involved in the variations of GnRH release during the estrous cycle in females and will be discussed further in the next chapter.

1.3.5 Peripheral factors

This chapter will exclusively focus on metabolic signals regulating puberty and reproduction. The hypothalamus acts as an integrator of peripheral signals regulating GnRH secretion. Many of those signals are metabolic factors involved in the cross-talk between the control of energy balance and reproduction.

Leptin is a hormone released by fat cells in the adipose tissue. It is an anorexigenic factor (reducing appetite and energy expenditure) signaling to the ARC in the hypothalamus. (Meister, 2000). *ob/ob* mice lacking the gene coding for leptin display obesity (Klok, Jakobsdottir, and Drent, 2007; Zhang et al., 1994) and do not go through puberty. In contrast, ghrelin, a hormone produced by the digestive system, is orexigenic and increases appetite (Cummings and Overduin, 2007).

The opposite effect of leptin and ghrelin on the control of energy balance is also found at the level of the reproductive function. Leptin is required for pubertal onset as leptin deficient mice are infertile and do not undergo puberty (Farooqi, 2002; Halaas et al., 1995). In our laboratory, leptin stimulates GnRH secretion from hypothalamic explants incubated *ex vivo* during the prepubertal period (Lebrethon et al., 2007). Accordingly, *in vivo* peripheral injection of leptin in undernourished mice and humans restores normal pubertal onset (Cheung et al., 2001; Donato et al., 2011a). Ghrelin exerts an opposite effect on pubertal onset. In rodents, *ex vivo* or *in vivo* administration of ghrelin during the prepubertal period decreases GnRH frequency and delays sexual maturation (Lebrethon et al., 2007; Fernández-Fernández et al., 2005; Martini et al., 2006; Tena-Sempere, 2008). While it is clear that leptin and ghrelin regulate the neuroendocrine system leading to pubertal onset, such effect do not seems to involve a direct action on GnRH neurons. GnRH do not express LEPR and ghrelinR (Hakansson et al., 1998; Odle et al., 2018). In the hypothalamus, several neuronal subtypes were found to express receptors for leptin and ghrelin and display transsynaptic connections with GnRH neurons. Pro-opiomelanocortin (POMC) neurons and neurons expressing both agouti-related protein (AgRP) and neuropeptide Y (NPY) appear to be the principal mediators of the interaction between leptin and GnRH secretion. POMC deficient mice display obesity and decreased fertility (Butler and Cone, 2002; Cone, 2005; Huszar et al., 1997). Along the same line, AgRP and NPY decrease GnRH frequency *ex vivo* or *in vivo* (Lebrethon et al., 2007; Catzeflis et al., 1993). Ghrelin increases *Npy* mRNA (Kamegai et al., 2001) and decreases *Kiss1* mRNA expression in the hypothalamus, while leptin induces the opposite effect (Tena-Sempere, 2006). However, mice lacking NPY or its receptor have normal pubertal development (Donato et al., 2011a). In addition, deletion of leptin re-

ceptors in hypothalamic kisspeptin neurons in mice does not affect or fertility (Donato et al., 2011b). A novel polypeptide, pituitary adenylate cyclase activating polypeptide (PACAP) was recently proposed to be the relay of nutritional information regulating GnRH release through the action on kisspeptin neurons (Ross et al., 2018). Overall, this data suggests the presence of a redundant network integrating the regulation of the GnRH network by metabolic signals.

Another metabolic factor stimulating GnRH secretion is cocaine- and amphetamine-regulated transcript (CART). CART-containing neurons are located in the ARC and the AVPV. They transmit satiety signal and decrease food intake (Abbott et al., 2001; Rondini et al., 2004; Kong et al., 2003). Most of ARC CART neurons are in close apposition with GnRH neurons in the mPoA and AVPV CART neurons are in close contact with KNDy neurons in the ARC (True et al., 2013). Electrophysiological studies suggested that CART can indirectly increase GnRH secretion by a postsynaptic depolarization of ARC KNDy neurons or by direct depolarization of GnRH neurons (True et al., 2013).

1.4 The GnRH network: transcriptional control of GnRH secretion

The increase in GnRH secretion characterizing pubertal onset requires coordinated changes in the transsynaptic neuron-to-neuron and glia-to-neuron interactions associated with GnRH neuron. These changes require the increase of stimulatory factors (i.e. kisspeptin, glial factors) and a decrease in transsynaptic inhibitory influences (i.e. GABA) in the hypothalamus throughout the prepubertal period. This switch is tightly controlled by genetic factors and is suspected to be redundant, ensuring the activation of the GnRH network and ultimately leading to pubertal onset (Lomniczi et al., 2013a). For instance, loss of *Gpr54* leads to pubertal failure but if *Kiss1* is suppressed during early development puberty is restored (Roux et al., 2003; Seminara et al., 2003). A similar compensatory mechanism appears in the absence of the NKB receptor, *Tac3r* (Yang et al., 2012). More recently, several genes coding for transcription factors playing a key role in the activation of GnRH secretion at puberty have been identified. *Eap1*, *Mkrn3*, repressive transcription factors as the zinc-finger gene *Znf462* and *Znf483*, the homeodomain *Ttif*, the Pou-domain gene *Oct2* and *Lin28b*. Enhanced at puberty

1 (*Eap1*) is one of the upstream transcriptional regulators of pubertal onset (Heger et al., 2007; Mastronardi et al., 2006). In rodents and rhesus monkey, deficiency in *Eap1* delays puberty and estrous cyclicity. The transcriptional factor encoded by *Eap1* transactivates the 5' regulatory region of the *GnRH* gene and inhibits preproenkephalin, a repressor of GnRH secretion. The stimulatory role of *Eap1* on GnRH transcriptional activity appears to be independent of *Kiss1* and gonadal steroids (Li and Li, 2017; Matagne et al., 2009). In a recent study, humans with delayed puberty were found to display two mutations in the *Eap1* gene (Mancini et al., 2019). The identified variants of *Eap1* showed a reduced ability to transactivate the GnRH promoter. Makorin RING-finger protein 3 (*Mkrn3*) is a maternally imprinted gene found to be mutated in around 30% of cases of familial central precocious puberty (CPP) (Abreu et al., 2015; Grandone et al., 2017; Neocleous et al., 2016). In rodents, hypothalamic *Mkrn3* expression decreases throughout sexual maturation (Liu, Kong, and Chen, 2017), suggesting an inhibitory role in pubertal onset. The developmental decrease in *Mkrn3* expression has been recently shown to implicate repression of the non-coding RNA mir-30 (Heras et al., 2019). The homeodomain thyroid transcription factor 1 *Ttf1* is involved in pubertal onset as well. Deletion of *Ttf1* in mice delays pubertal onset and disrupt estrous cyclicity (Mastronardi et al., 2006).

In humans, pubertal timing is highly variable among individuals. Around 60-80% of this variance depends on genetic factors (Heger et al., 2007; Parent et al., 2003; Palmert and Hirschhorn, 2003; Towne et al., 2005; Zhu, Kusa, and Chan, 2018).Genome-wide association (GWAS) studies in humans (He et al., 2009; Ong et al., 2009; Perry et al., 2009; Sulem et al., 2009), have provided the first evidence of common genetic variants related to puberty. In a more recent study, 386 loci were identified to influence pubertal timing among more than 38,000 women (Day et al., 2017). However, the identified loci were found to account only for 7.4% of the variance, suggesting the presence of other variants yet to be discovered or the existence of mechanisms other than single-nucleotide polymorphism such as copy number variations or epigenetic changes that could account for variance in pubertal timing (Zhu, Kusa, and Chan, 2018).

While a series of genes critical for pubertal success have been discovered, little is known about their transcriptional weight in triggering puberty and how those

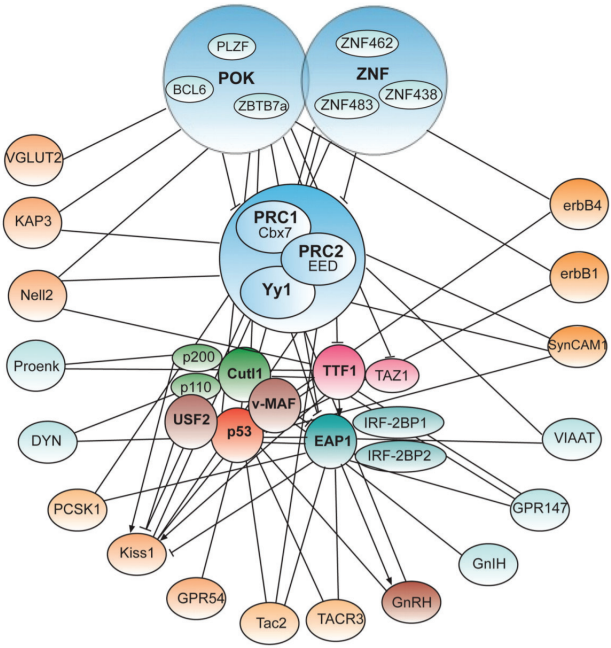


Figure 1.6. Predicted model of the transcriptional control of pubertal onset. In this model, two upstream nodes containing the transcriptional repressors *Pok* and *Znf* inhibit the PcG chromatin remodelers. PcG are silencing complex repressing gene transcription. Removing PcG expression during development leads to the activation of genes in a downstream subordinate level such as *Kiss1*, *GnRH* and *SynCam1* promoting pubertal onset. Obtained with permissions from Lomniczi et al., 2013a.

factors relate to each other. Known gene mutations account only for a small number of individuals presentin abnormal in pubertal development, suggesting that puberty is not triggered by a single gene but rather a network of interconnected genes. For instance, the developmental switch from inhibition to activation of the GnRH network is now known to require the transcriptional synchronization of hundreds of genes arranged in interconnected hubs at different functional hierarchical levels (Lomniczi et al., 2013a) (Fig.1.6). Using a system biology approach, Lomniczi et al. (2013) have shown the existence of a core of transcriptional regulators orchestrating expression of subordinate genes that activate puberty. Using rat and rhesus monkey MBH explants and a high-throughput genomic and proteomic approach at three developmental stages (juvenile, early puberty and adult-

hood), 2 modules of strongly interconnected genes activating or repressing peripheral nodes were identified. In these two nodes, *Cutl1/p53*and *Ttf1/Eap1* function were central hubs, controlling peripheral subordinate genes such as *Kiss1*, *SynCam1*, *Rfrp3* and GnRH itself. *SynCam1* is involved in the developmental control of glia-to-neuron communication in the hypothalamus facilitating GnRH secretion (Sandau et al., 2011a; Sandau et al., 2011b; Ojeda, Lomniczi, and Sandau, 2008). *In silico* models have predicted that *Kiss1* expression is controlled by *Ttf1*, *Eap1*, *Cutl1* and *Yy1*, supporting this model (Mueller et al., 2011). In addition, these two central nodes were predicted to be regulated by two supplementary nodes functionning as upstream modulators of the central hub. For instance, a node composed by *Pok* and zinc finger protein genes suppresses the action of the repressor polycomb group (*PcG*) of chromatin remodelers. Overall, this model predicts that the upstream POK/ZNK proteins repress PcGs proteins, which in turn remove the brake to the subordinate activating pubertal genes.

1.5 The GnRH network: the novel role of epigenetics in the onset of puberty

Some of the upstream components of the network discussed in the previous section, such as the PcG proteins (Lomniczi et al., 2013b), are known to be epigenetic regulators of gene expression (Ball et al., 2009; Guenther et al., 2007; Koch et al., 2007; Miao and Natarajan, 2005; Mikkelsen et al., 2007). The role of chromatin remodeling factors in pubertal onset has only been addressed in recent years. In the next sections, the main mechanisms of epigenetic regulation of gene expression will be briefly discussed before introducing the state of the art in the epigenetic control of pubertal onset.

1.5.1 Epigenetic regulation of gene expression

While the concept of inheritance of acquired traits dates back to ancient Greece, one of the most remarkable precursors of the modern concept of epigenetics was Jean-Baptiste Lamarck. The French naturalist "primitively" postulated around the year 1800 that "*changes in animal organization produced by the movement of fluids internal to the animal body were conserved and transmitted successively by generation*" (de Monet de Lamarck, 1802). His works were likely inspired by those

of LeRoy (Leroy, 1802) and Condorcet (Condorcet, 1794) who believed in the inheritance of acquired traits based on their observation of wildlife (Burkhardt Jr, 2013). However, these ideas were abandoned by western scientist, especially during the advent of mendelian genetics and Darwinian theory of evolution (Deichmann, 2016). In a more contemporary approach, Paul Kammerer published in 1924 a series of controversial experiments carried out in midwife toads (a genus of frogs) in a book entitled "The inheritance of acquired characteristics" that were later discredited by accusations of fraud (Kammerer, 1924; Vargas, 2009). Despite several attempts to describe what we call today epigenetics during the last centuries, the foundation of the field is often exclusively credited to Conrad Waddington. In 1942, this embryologist described the role of environmental factors in the heritable variations in thorax and wing structure in fruit flies (Waddington, 1942). The discovery of different phenotypes in subjects with the same genetic background (genotype), led him to propose a definition of epigenetics, as "...the interaction of genes with their environment which brings the phenotype into being" (Waddington, 1942).

In modern terms, epigenetic is the field that studies "the heritable phenotype resulting from changes in a chromosome structure without alterations in the DNA sequence" (Berger et al., 2009). In other words, the changes in gene expression by the action of heritable and reversible chemical modifications of chromatin without changing the DNA sequence itself. In eukaryotic cells, DNA is packed in the nucleus wrapped around histone octamers to form the chromatin structure. In genome regions where chromatin is more condensed (known as heterochromatin) transcription is inactive as DNA is inaccessible. When chromatin is in a open state, the transcriptional machinery has free access to these DNA regions (known as euchromatin). Epigenetic regulation of gene expression occurs through the modification of chromatin states in specific regions of the genome. Such modifications involve chromatin remodeling proteins that alter the chemical composition of histones or DNA itself. The three epigenetic modifications described so far comprise post-translational modification (PTM) of histone tails, DNA methylation and the indirect action of non-coding RNA (ncRNA). These mechanisms contribute to the dynamic changes in chromatin structure and DNA accessibility regulating gene expression.

1.5.1.1 DNA methylation

DNA methylation consists in the covalent addition of a methyl group to the C5 position of a cytosine, often adjacent to a guanine (so called CpG)(Fig.1.7.A). CpG-rich regions (also known as CpG islands) are often found in regulatory regions of the genome. The addition of the methyl group is catalyzed by the enzyme DNA methyltransferase (DNMT) resulting in 5-methyl cytosine (5-mC). DNMTs are provided by the methyl donor S-adenosyl methionine (SAM). The 5-mC is often associated with gene repression as its presence promotes the recruitment of methyl-binding proteins, which in turn recruit histone deacetylases (HDACs) that ultimately induce heterochromatin formation (Jones and Takai, 2001; Hashimoto, Vertino, and Cheng, 2010). When methylation occurs in CTCF binding sites, typically found in insulator regions between promoter and enhancer regions, DNA methylation increases gene expression (Lai et al., 2010). DNA methylation is a reversible process. The enzymes ten-eleven translocation methylcytosine dicoxygenases (TET) oxydates 5-mC into 5-hydroxymethylcytosine (5hmC). The 5hmC is associated with active transcription and appears to be often present in gene bodies (Ficz et al., 2011; Guo et al., 2011; Tahiliani et al., 2009).

DNA methylation is mitotically heritable, which requires the action of DNMT1 to ensure the preservation of 5-mC throughout cell replication. DNMT1 is vital for development as deficiency of *Dnmt1* leads to embryonic lethality (Li, Bestor, and Jaenisch, 1992), while DNMT3a and DNMT3b catalyze *de novo* formation of 5-mC (Smith and Meissner, 2013). DNMT and TET enzymes are known to interact with nuclear receptor transcription factors such as the estrogen receptor beta (ERβ) to regulate DNA methylation at specific loci (Liu et al., 2016).

DNA methylation involved in the sexual differentiation of the mPoA through the regulation of estrogen receptor alpha (ERα) (McCarthy and Nugent, 2013; Kurian, Olesen, and Auger, 2010). In the AVPV, higher levels of DNA methylation are found at CpG sites in the *Kiss1* gene promoter in females compared to males. In this region, AVPV *Kiss1* expression is higher in females, suggesting that methylation may occur in an insulator region (Semaan et al., 2012).

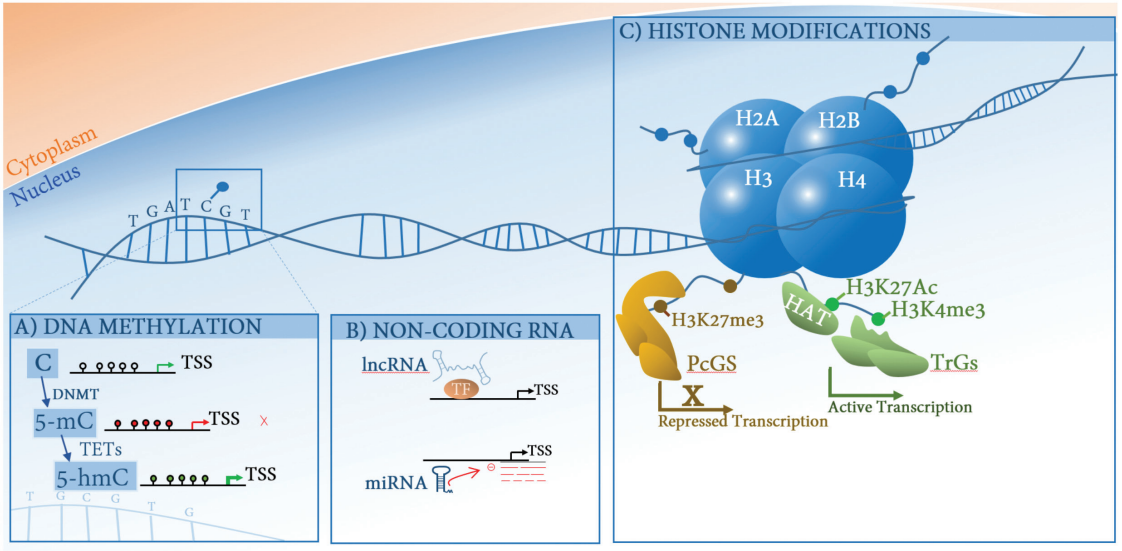


Figure 1.7. Mechanisms of epigenetic control of gene expression. (A) CpG methylation is catalyzed by the action of DNA methyltransferases (DNMT) leading to the production of 5-methylcytosine (5-mC). Such reaction can be reversed by the ten-eleven translocation family of proteins (TETs). (B) EDCs can alter the expression of long (lncRNA), small (sncRNA) and micro (miRNA) non-coding RNA. Non-coding RNAs are known to interact with specific epigenetic modifiers and direct DNMTs to specific loci. (C) Post-translational histone tail modifications (i.e. methylation, acetylation) regulate chromatin state, inducing or repressing gene transcription. Chromatin remodeling proteins, such as histone acetyltransferases (HAT), the polycomb (PcGs) or trithorax (TrGs) group of proteins, triggers such chemical modifications and have been recently shown to be involved in pubertal onset.

1.5.1.2 Non-coding RNAs

Only 1% of the genome is translated into proteins (Lee, 2012)(Fig.1.7.B). Among the rest, 70-90% of the genome translate into RNA transcripts not coding for proteins, also called non-coding RNAs (ncRNA) (O'Carroll and Schaefer, 2013). These transcripts play an important role in regulating gene expression through a myriad of mechanisms. Different types of ncRNA have been identified, categorized by their size as small non-coding RNA (sncRNA) and long non-coding RNA (lncRNA). sncRNAs are sequences no longer than 200bp. They repress translation by mRNA degradation and specifically target mRNA of genes required for DNA methylation and histone PTMs (Gruber and Zavolan, 2013). Among the sncRNAs, micro RNA (miRNA) and piwi-interacting RNA (piRNA) have been described (Okamura et al., 2008; Kim, 2006). LncRNAs are RNA transcripts bigger than 200bp. They form complexes with proteins that regulate histone conformation.

1.5.1.3 Histone post-translational modifications (PTMs)

DNA is wrapped in an octamer of histones creating a structure called nucleosome (Fig.1.7.C). Histone octamers are formed by pairs of the histone subunits H2A, H2B, H3 and H4. The additional histone H1 acts as a stabilizer linking all subunits. Chromatin state is determined by the electrical binding affinity between histones and DNA. It is modulated by PTM at the N-terminal of histone tails (Bannister and Kouzarides, 2011; Gillette and Hill, 2015).

The most studied histone PTMs are acetylation and methylation but PTMs also include phosphorilation, ubiquitination and sumoylation, among others (Kouzarides, 2007; Khorasanizadeh, 2004). Such modifications are dynamic, reversible and play different functional roles in gene regulation. The post-translational addition of an acetyl group at lysine of histone tails always induces transcriptional activation by changing chromatin conformation to an open state, while histone deacetylation is related to gene silencing and compacted chromatin (Kouzarides, 2007; Khorasanizadeh, 2004). Lysine methylation can activate or repress transcription depending on the position where it occurs and whether the lysine is mono-, di- or trimethylated (Gillette and Hill, 2015). For example, monomethylation of lysine 9 or 27 of the histone 3 (H3K9me and H3K27me) is linked to gene repression (Wang et al., 2008; Ruthenburg et al., 2007). However,

trimethylation of lysine 4 of histone 3 results in gene activation (Wang et al., 2008; Berger, 2007).

The role of histone modifications among the GnRH network has not been addressed yet but it is suggested by the existence of a sexual dimorphic histone PTM composition in different brain regions. For instance, male mice have higher levels of H3K9ac and H3K14ac in the cortex and hippocampus during late gestation until birth (Tsai, Grant, and Rissman, 2009). Males also show increased H4 acetylation in the mPoA (Matsuda et al., 2011). Moreover, late gestational administration of testosterone to females increases histone PTMs to male-like levels (Tsai, Grant, and Rissman, 2009). Peripheral administration or infusion of an HDAC inhibitor into the mPoA to males at birth alters adult male reproductive behavior and eliminates sex differences in the bed nucleus of the stria terminalis (BNST) (Matsuda et al., 2011; Murray et al., 2009). Level of the histone methyl transferase (i.e. MLL1) and histone demethylases (KDM5c) were also found to be sexually dimorphic in other brain regions (Huang et al., 2007; Xu, Deng, and Disteche, 2008). These data suggest that variations in histone marks in the brain may be modulated by hormonal levels and that they may be critical for brain sexual differentiation and reproduction.

1.5.1.4 Chromatin remodeling

The epigenetic control of gene expression is a very dynamic and synchronized process that involves all three epigenetic mechanisms: DNA methylation, histone PTMs and ncRNAs. This process is regulated by proteins called chromatin remodelers (Gillette and Hill, 2015; Cameron et al., 1999; Cedar and Bergman, 2009). We can distinguish three categories depending on whether they recognize a chromatin structure (readers), add (writers) or remove (erasers) chemical modifications. The best characterized chromatin remodelers are those involved in DNA methylation (DNMTs), in histone acetylation/methylation (writers): the histone acetyl transferases (HATs) and histone methyl transferases (HMTs); or in deacetylation/demethylation (erasers), the HDACs and histone demethylases (HDMs), respectively. Interaction between chromatin remodelers and epigenetic marks has been described. To cite a few examples, DNMTs associate with HDACs to induce gene repression (Rountree, Bachman, and Baylin, 2000; Robertson et al., 2000; Burgers, Fuks, and Kouzarides, 2002) and H3K9me facilitates DNMT3a

and DNMT3b recruitment at gene regulatory regions (Cedar and Bergman, 2009). Finally, readers recognize specific histone PTMs or other chromatin remodelers, such as writers and erasers, to direct a particular transcriptional outcome (Yun et al., 2011).

Chromatin remodelers are found in functional complexs such as switching defective/sucrose non/fermenting (SWI/SNF), polycomb group of proteins (PcG) or trithorax group of proteins (TrxG). PcG and TrxG were recently found to be involved in female sexual maturation (Lomniczi et al., 2013b; Toro et al., 2018; Lomniczi et al., 2013a). The polycomb group (PcGs) is a group of epigenetic repressors composed of two complexes: PRC1 and PRC2. The PRC2 complex is recruited by specific polycomb response elements catalyzing H3K7me3, a repressive histone modification (Simon and Kingston, 2009). To induce a homogeneous response, the CBX proteins of the PRC1 complex bind to H3K7me3 reproducing the repressive histone mark in the genomic loci (Simon and Kingston, 2009; Schwartz and Pirrotta, 2007; Otte and Kwaks, 2003). PcG complexes has specific functions during embryonic development and in pluripotent stem cells (Aloia, Di Stefano, and Di Croce, 2013).

The trithorax (TrxG) group of proteins counteracts PcGs repression of gene transcription (Schuettengruber et al., 2011; Shilatifard, 2012). There are six protein complexes termed COMPASS and COMPASS-like. The COMPASS complex contains SET1A and SET1B and leads to H3K4me3, histone mark associated with active promoter regions (Hu et al., 2013). Among the COMPASS-like, MLL1/MLL2 are related to *TrxG* and mediate the action of several active histone PTMs at bivalent promoters (Bernstein et al., 2006). Two other COMPASS-like complexes contain MLL3/MLL4, related to *Trr* implementing H3K4me at gene enhancer regions (Hu et al., 2013).

1.5.2 The epigenetic control of puberty

Only a few studies have addressed the question the epigenetic control of puberty. Using whole-genome bisulfite sequencing in goats, it was demonstrated that DNA methylation changes are required for pubertal onset (Yang et al., 2016). In addition, DNA methylation of the *GnRH* promoter developmentally decreases in rhesus monkeys, allowing transcription at the time of puberty (Kurian, Keen, and

Terasawa, 2010). The role of non-coding RNAs in puberty has only been addressed recently. Hypothalamic expression of the miRNA let-7 and lin-28 was found to be necessary for pubertal onset in rats and monkeys (Sangiao-Alvarellos et al., 2013). Furthermore, mir-30, mir-200 and mir-155 ware found to be critical for pubertal onset by repressing *Mkrn3*, *Zeb1* and *Cebpb* (Heras et al., 2019; Messina et al., 2016).

The most comprehensive studies to date on the topic were recently published by the Lomniczi team, describing the role of three major groups of chromatin remodelers expressed in the hypothalamus, directing the developmental transcriptional changes at the GnRH network that drives puberty (Lomniczi et al., 2013b; Lomniczi et al., 2013a; Toro et al., 2018; Lomniczi et al., 2015). In rats, high levels of the repressive PcGs members, *Eed* et *Cbx7* and its associated repressive histone PTMs marks, are present at the level of the *Kiss1* gene promoter in the ARC (Lomniczi et al., 2013b) (Fig.1.8). PcGs presence at the *Kiss1* promoter decreases right before puberty. , Simultaneously, expression of active histone marks increases. This change in histone landscape facilitates the increase in ARC *Kiss1* expression at puberty.

Open chromatin state at the *Kiss1* promoter is triggered by the increase in TrxG proteins, *Mll1* and *Mll3* at the promoter and enhancer region of *Kiss1*, respectively (Toro et al., 2018). Targeted deletion of *Mll1* in ARC Kiss neurons, decreases H3K4me3 at the *Kiss1* promoter region, inhibiting gene expression. Importantly, inactivation of *Mll1* or diminished binding of MLL3 to the *Kiss1* enhancer region leads to pubertal and reproductive failure. In the previous section, we summarized the role of two predicted upstream nodes regulating subordinate genes controlling puberty (Lomniczi et al., 2013a). Among the genes in that node, zinc finger proteins were postulated as "repressor of repressors" of puberty. The members of the zinc finger family GATAD1/KDM1A appeared to be involved in the reawakening of the GnRH network in rhesus monkeys (Lomniczi et al., 2015). At the time of puberty, expression of repressor GATAD1 decreased in the hypothalamus. Overexpression of GATAD1 in the ARC of rats, removes the activational H3K4me2/3 histone marks at the *Kiss1* and *Tac3* gene promoter and delays puberty. The effect of GATAD1 on histone PTMs is mediated by the recruitment of the eraser KDM1A. The aforementioned results imply that in ARC

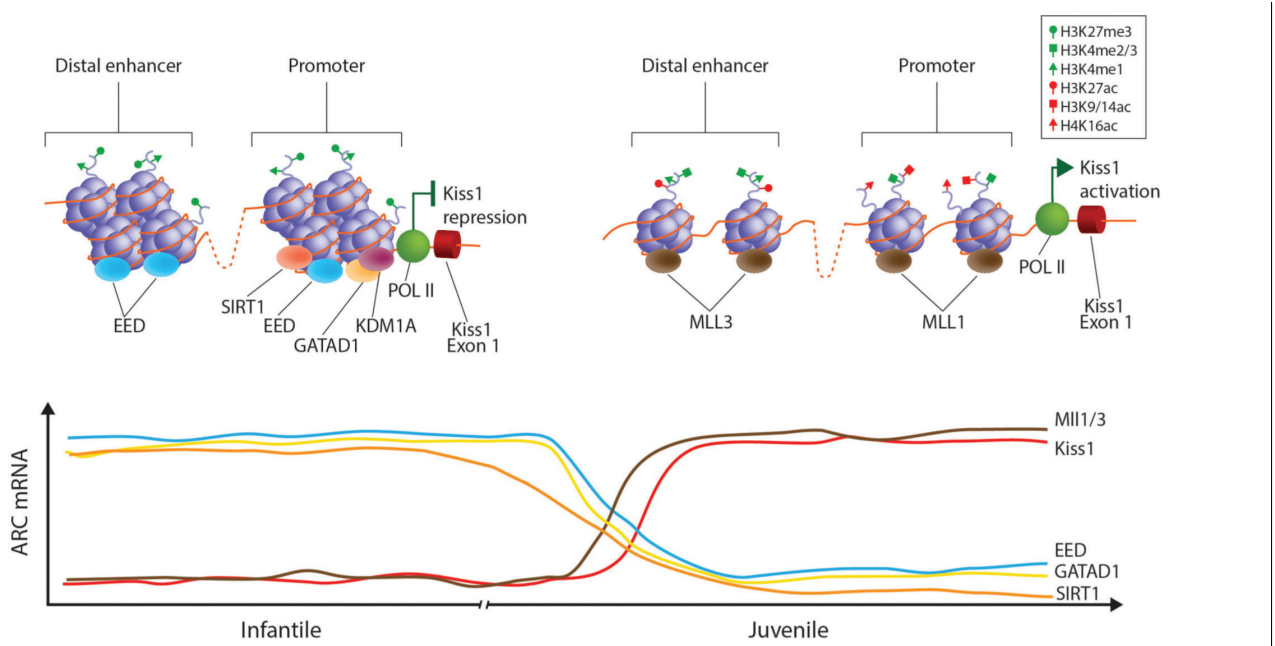


Figure 1.8. Model of the developmental epigenetic changes at the *Kiss1* promoter controlling its transcription from the juvenile to pubertal onset. During the infantile period, the GnRH network is quiescent. At the *Kiss1* promoter, expression of chromatin repressors (EED, SIRT1, GATAD1 and KDM1A) and repressive histone marks are highly expressed. This repressive epigenetic landscape ensure low *Kiss1* mRNA levels. At puberty, expression of chromatin repressors at the promoter and enhancer is replaced by TrxG proteins (MLL1 and MLL3) and active histone marks that trigger an open chromatin state and active transcription of *Kiss1*. Obtained with permissions from Lomniczi, Aylwin, and Vigh-Conrad, 2019.

KNDy neurons there is a balance between activating and repressing epigenetic factors that play a role in the correct timing of *Kiss1* expression at puberty. Overall, these studies demonstrate a functional link between different members of the epigenetic machinery and the activation of the GnRH network.

2 The neuroendocrine control of ovulation

Contents

This chapter will focus on the neuroendocrine control of the female reproductive system in rodents. The reawakening of the GnRH pulse generator during puberty allows the peripheral release of ovarian hormones and the onset of reproductive maturation. In female rodents, onset of the reproductive life is characterized by the increase of ovarian hormones and the stimulation of follicle maturation, which triggers the first ovulation and the beginning of estrous cyclicity. In the next sections we will briefly describe oogenesis which start during fetal life and the ovarian events taking place during the estrous cycle, such as steroidogenesis and folliculogenesis. Thereafter, the next sections will describe the central factors involved in the hypothalamic control of ovulation.

2.1 Oogenesis and ovarian events during the estrous cycle

In rodents, the estrous cycle is characterized by four phases: metestrus, diestrus, proestrus and estrus (Butcher, Collins, and Fugo, 1974) (Fig.2.1.C). During metestrus, progesterone and estradiol levels are low. During diestrus progesterone levels start to increase dropping at the end of the day. In the afternoon of proestrus, estradiol and progesterone levels rise, causing a rapid surge in gonadotropins called the preovulatory surge. During the estrus stage, ovulation occurs and hormone levels return to baseline by the evening of estrus (Butcher, Collins, and Fugo, 1974). In rodents, a complete regular estrous cycle lasts 4 to 5 days.

The hormonal changes during the estrous cycle depend on the communication between central and peripheral signals. Hypothalamic gonadotropin release regulates steroidogenesis and follicular development in the ovaries. In the next sections, we will briefly summarize the process of oogenesis followed by the mechanisms of ovarian follicle formation and steroidogenesis.

2.1.1 Oogenesis

Oogenesis is the process that encompasses the birth of a germ cell, its migration, differentiation, and growth by interacting with somatic cells (i.e. ovarian granulosa cells) to be ultimately assembled as ovarian follicles (Fig.2.1.A). Oogenesis involves the maturation of a pluripotent cell able to propagate into the next generation.

The primordial germ cells (PGCs), precursors of oocytes, arise in the yolk sac around embryonic day 5 (Wear, McPike, and Watanabe, 2016). At the same time that they undergo mitosis, PGCs travel a long distance through the hindgut and the gonadal ridge to their final destination in the gonads around embryonic day 12.5 in mice (Saitou, 2009). During the migration and colonization of the gonads, PGCs sexually differentiate into oocytes and undergo rapid mitosis. At E12.5 there are around 3,000 PGCs. This number rises to 18,000 at E14.5 (Lei and Spradling, 2013). However, the number of PGCs rapidly decreases from apoptosis from 18,000 at E18 to 8000 at postnatal day 2 (Lei and Spradling, 2013). During

this period of oocyte atresia, the remaining germ cells cluster in nests surrounded by somatic granulosa cells. At birth, estradiol concentrations decrease, inducing the breakdown of germ cell nests. Granulosa cells encircle individual oocytes by a single layer of cells, forming the primordial follicles (Tingen, Kim, and Woodruff, 2009). One third of the oocytes are lost during nest breakdown (Pepling and Spradling, 2001; Bristol-Gould et al., 2006).

Primordial follicles formed during early postnatal life constitute the pool of potentially fertilizable eggs through the female lifespan. However, recent studies have documented the formation of stems cell in the ovary months after birth (Johnson et al., 2004; Imudia et al., 2013; Niikura, Niikura, and Tilly, 2009). These controversial studies contradict others (Zhang et al., 2014; Zhang et al., 2012) but it appears that these adult generated stem cells could contribute to the germ cell pool (Zou et al., 2009).

2.1.2 Folliculogenesis

The primary functions of the ovary are to synthesize sex steroids and mature follicles to release a fertilizable egg (Freeman, 2006) (Fig.2.1.A). Ovarian folliculogenesis starts with primordial follicles, an oocyte surrounded by a single layer of squamous granulosa cells. Starting at puberty, primordial follicles may be activated to undergo a series of developmental transitions known as ovarian folliculogenesis. Alternatively, follicles can remain quiescent or undergo atresia. During primordial follicle recruitment, oocytes increase in size and the single layer of granulosa cell becomes cuboidal in shape. This now primary follicles are recruited into secondary follicles, containing at least two layers of granulosa cells and an external layer of theca cells (Freeman, 2006).

When secondary follicles mature, increasing in size and contain a fluid-filled space, they are called antral follicles (Freeman, 2006; Zeleznik, 2004). These mature follicles are recruited in every estrous cycle to undergo ovulation by releasing its oocyte. The remaining somatic cells of the follicle that lead to ovulation differentiate into luteal cells to become a structure called corpora lutea. The presence of corpora lutea in the ovaries serves as a marker of ovulation and fertility.

Because of the presence of theca and granulosa cells and their level of maturation, secondary follicles are gonadotropin-responsive and able to release sex

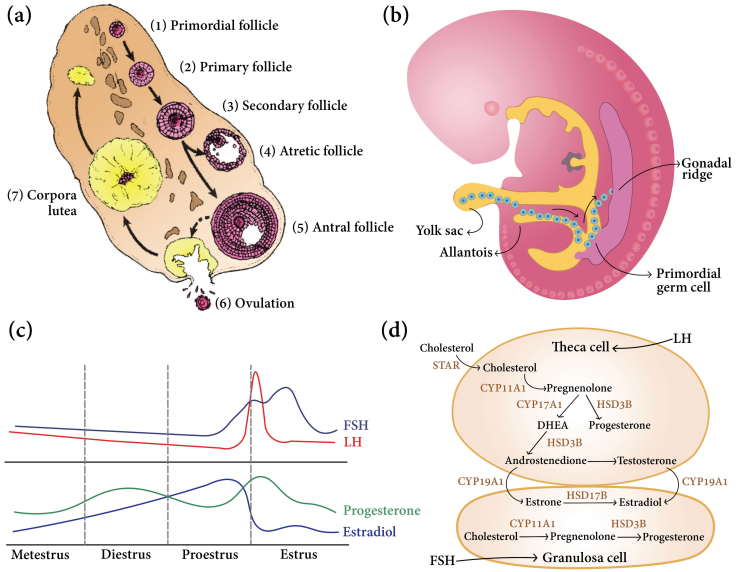


Figure 2.1. Developmental origin of ovarian reserve and hormonal events during estrous cycle in the rat. (a) Morphology and development of ovarian follicles from primary to corpus luteum. A single antral follicle is recruited to produce ovulation at the estrus stage, releasing the oocyte. (b) Representation of germ cell migrations from the fetal yolk sac to the gonadal ridges by the mesentery. (c) Serum levels of progesterone, estradiol, LH and FSH during the four stages of the estrous cycle in the rat: estrus, diestrus 1 (metestrus), diestrus 2, proestrus and estrus. (d) Ovarian steroidogenesis. Testosterone and estradiol are synthesized by enzymatic conversion from cholesterol at theca and granulosa cells, respectively.

steroids (Freeman, 2006; Zeleznik, 2004). Ultimately, from the initial pool of follicles, only a small fraction follows recruitment and maturation. Thus, most of follicles will undergo atresia, a process of degeneration by apoptosis that affects the somatic cells and the oocyte. Because of their large number and their unresponsiveness to LH and FSH, primary follicles are more likely to undergo atresia. The presence of antiapoptotic factors as gonadotropins, estrogens and IGFα helps to prevent atresia in late stage follicles (Braw, Bar-Ami, and Tsafriri, 1981; Braw and Tsafriri, 1980; Terranova, 1981; Billig, Furuta, and Hsueh, 1993; Chun et al., 1994).

2.1.3 Ovarian steroidogenesis

The follicle ability to produce sex steroids starts with the secondary follicles as the presence of both granulosa and theca cells is required (Fig.2.1.D). Steroidogenesis that regulates female reproduction, is modulated by gonadotropins and involves the enzymatic conversion of cholesterol into estradiol. Cholesterol is synthesized by theca cells or transported into the cells by the enzyme steroidogenic acute regulatory protein (STAR) (Strauss et al., 1999; Christenson and Strauss, 2000). At the level of the mitochondria and endoplasmatic reticulum of theca cells, cholesterol follows a series of conversions into pregnenolone, DHEA, progesterone, androstenedione and ultimately, testosterone (Hanukoglu, 1992). Thereafter, testosterone is transported to granulosa cells where it is converted into estradiol by the action of the enzyme aromatase (or CYPA9A1) (Hayes et al., 1996). Estradiol synthesis depends on the coordination of granulosa and theca cells and the stimulatory action of centrally produced gonadotropins (Falck, 1959). The role of gonadotropins is crucial as theca and granulosa cells only respond to LH or FSH, respectively. For instance, LH stimulates the transcription of theca-derived genes encoding for enzymes critical for testosterone synthesis, whereas FSH stimulates transcription of granulosa-derived genes such as aromatase (Strauss et al., 1999; Christenson and Strauss, 2000; Falck, 1959).

2.2 Hypothalamic control of ovulation

During proestrus, the ovaries sustain an elevated synthesis of estradiol and progesterone, causing a switch in the central feedback from negative to positive (Moenter, Brand, and Karsch, 1992; Sarkar et al., 1976). In consequence, positive estrogen feedback induces a rise in GnRH secretion called the preovulatory LH surge, ultimately leading to ovulation. Classically, in rodents, the estradiol-induced LH preovulatory surge has been identified by using hormone manipulation studies (Dror, Franks, and Kauffman, 2013; Silveira et al., 2017). Estradiol replacement at low levels in ovarectomized females induces negative feedback, whereas injection to a high dose of estradiol induces a positive feedback (Bronson, 1981). Moreover, electrophysiological measures of GnRH GFP-labeled neurons showed that during the afternoon of proestrus these cells have a high firing rate (Christian, Mobley, and Moenter, 2005; Glanowska, Venton, and Moenter,

2012).

The estrogen positive feedback is primary mediated by ERα, despite of the participation of other estrogen receptors as ERβ, GPR30 and mER (Kuiper et al., 1996; Tremblay et al., 1997; Koike, Sakai, and Muramatsu, 1987; Micevych, Mermelstein, and Sinchak, 2017). Deficient ERα female mice (ERαKO) display no corpora lutea, high LH levels and reproductive failure (Lubahn et al., 1993). Deficiency in ERβ seems less critical, inducing a subfertile phenotype and normal LH levels (Krege et al., 1998). These studies suggest that ERα appears to mediate the positive feedback (Glidewell-Kenney et al., 2007) but few or no GnRH neurons express this receptor (Hrabovszky et al., 2000; Shivers et al., 1983). These neurons are known to express ERβ (Sharifi, Reuss, and Wray, 2002), GPR30 (Terasawa, Noel, and Keen, 2009), ERRγ (Klenke, Constantin, and Wray, 2016) and STX-sensitive membrane ER (Kenealy, Keen, and Terasawa, 2011). Estrogen positive feedback must be then mediated by intermediary neurons projecting onto GnRH neurons.

2.2.1 Kisspeptin control of the preovulatory LH surge

It is now widely accepted that the AVPV population of kisspeptin neurons function as a relay of the estradiol positive feedback to GnRH neurons (Smith et al., 2006; Smith et al., 2005a). This specific population of kisspeptin neurons, much larger in females, is organized by sex steroids neonatally (Navarro et al., 2009b; Kauffman et al., 2007b; Navarro et al., 2004). It has been recently demonstrated that mERα (Khbouz et al., 2019) is involved in the sexual differentiation of the male brain by perinatal estrogen (Patisaul and Adewale, 2009). Female mice neonatally treated with estradiol benzoate (Khbouz et al., 2019) or testosterone (Kauffman et al., 2007b) had a profile of kisspeptin expression similar to males and estradiol has been shown to be unable to evoke a positive feedback after neonatal androgenization of female rats (Kauffman et al., 2007b). Thus, ERα appears to play a predominant role in the masculinization of the AVPV kisspeptin neurons.

More than 70% of AVPV kisspeptin neurons express ERα and at least a third has direct transsynaptic connections with GnRH neurons (Kumar et al., 2015; Kauffman et al., 2007b; Clarkson and Herbison, 2006b). Moreover, these neurons are activated during the LH preovulatory surge as suggested by the activation of

cfos at the time of the LH surge (Clarkson and Herbison, 2006b; Clarkson et al., 2008; Le et al., 1999). In agreement with these results, electrophysiological studies showed that action potential in AVPV kisspeptin GFP-labeled neurons increase in frequency during proestrus compared to diestrus (Wang, DeFazio, and Moenter, 2016; Piet, Boehm, and Herbison, 2013). In a recent study, selective optogenetic activation of AVPV kisspeptin neurons induced an increase in LH secretion at levels similar to those found during the LH surge (Piet et al., 2018). Additionally, during proestrus, AVPV kisspeptin neurons receive increased glutamate and decreased GABAergic input (Wang et al., 2018; DeFazio, Elias, and Moenter, 2014).

2.2.2 Circadian control of the preovulatory LH surge

The suprachiasmatic nucleus (SCN) located in the anterior hypothalamus orchestrates circadian rhythms (Moore and Eichler, 1972; Stephan and Zucker, 1972). In mammals, this region generates endogenous rhythms based on sensory information received by the retina. The SNC is now known to communicate with the GnRH network. For instance, mouse clock mutants, which have an ablation of the SCN display a disruption of estrous cycle and pregnancy failure (Miller et al., 2004).

During proestrus, SCN activation precedes the preovulatory LH surge (Legan, Coon, and Karsch, 1975; Mahoney et al., 2004) and requires high levels of estradiol (Kriegsfeld and Silver, 2006; Legan, Coon, and Karsch, 1975; Christian and Moenter, 2010). In this region, a small number of neurons expresses ERα, whereas ERβ is highly expressed and exhibits a circadian rhythm pattern of expression (Vida et al., 2008; Wilson et al., 2002). Moreover, SCN sends direct projections to the AVPV (Iglesia, Blaustein, and Bittman, 1995; Watson et al., 1995) where multiple factors triggering ovulation converge. Among the cell populations found in the SCN, vasopresin neurons play a major role in the control of ovulation. These specialized SCN neurons release vasopressin peptide in a circadian manner (Shinohara et al., 1994), directly targeting the AVPV (Watson et al., 1995), a step ultimately required for the LH surge (Palm et al., 1999).

Kisspeptin neurons receive and integrate circadian output signals to trigger the preovulatory LH surge. AVPV kisspeptin cells show increased *cfos* mRNA expression during the afternoon of proestrus (Smith et al., 2006; Adachi et al.,

2007), a change in expression that is abolished after ovariectomy (Robertson et al., 2009). Kisspeptin neurons receive direct input from vasopressinergic SCN neurons (Vida et al., 2010), controlling their circadian activity (Williams et al., 2011). AVPV administration of vasopressin induces the release of kisspeptin at levels similar to those found during the preovulatory LH surge (Williams et al., 2011).

At the molecular level, SCN cells generate a circadian rhythm by autoregulatory feedback loops orchestrated by a serie of clock genes (Reppert and Weaver, 2002; Maywood et al., 2007; Chen et al., 2009; Mohawk and Takahashi, 2011). The heterodimerization of the clock circadian regulator (*Clock*) and aryl hydrocarbon receptor nuclear translocator-like 1 (*Bmal1*) genes promotes the transcription of the period genes (*Per1*, *Per2* and *Per3*) and cryptochrome (*Cry1*, *Cry2*) genes. Thereafter, *Per* and *Cry* transcripts accumulate during the diurnal phase of the day forming hetero and homodimers to, in turn, inhibit *Clock* and *Bmal1* transcription. SCN expression of clock genes is critical for reproductive function. *Clock* deficiency leads to alterations in estrous cyclicity and reproductive failure (Miller et al., 2004). While the SCN is the main pacemaker, expression of *Clock* and *Bmal1* have been found in other regions of the brain. Importantly, GnRH neurons express clock genes, having their own internal pacemaker (Olcese et al., 2003; Hickok and Tischkau, 2010; Matagne et al., 2012).

2.2.3 Role of glia in GnRH variations during the estrous cycle

Astrocytes and tanycytes, a unique type of ependymoglial cells found in the third ventricle wall, selectively interact with GnRH terminals at the level of the median eminence (Kozlowski and Coates, 1985; Clasadonte and Prevot, 2018). These glial cells dynamically control GnRH release variations during the estrous cycle (Clasadonte and Prevot, 2018).

During diestrus, the semaphorin 7A (SEMA7A) is secreted by tanycytes in response to high progesterone levels (Parkash et al., 2015). SEMA7A activates Integrin β1, which in turn promotes the growth of tanycytic end feet, blocking the capillary that surround GnRH terminals. This mechanism prevents GnRH to be released into portal circulation (Parkash et al., 2015). Concomitantly, SEMA7A activates Plexin C1 in GnRH terminals which leads to their retraction (Parkash et

al., 2015). During proestrus, circulating NO increases in response to high estrogen levels and activates the synthesis of semaphorin 3A (SEMA3A). By its action on GnRH neurons through the NRP1 receptor, SEMA3A induces the GnRH terminals growth in order for them to be in close contact with endothelial cells of the portal system (Giacobini et al., 2014; Seranno et al., 2010; De Seranno et al., 2004), allowing GnRH to be released at the time of the preovulatory LH surge.

3 Introduction to Endocrine Disrupting Chemicals (EDCs)

Contents

The environment is a modulatory factor able to interfere with the development of an organism. Nutrition, physical activity or exposure to chemicals are environmental factors abe to alter an organism trajectory. They are known to influence the neuroendocrine system and, by consequence, have an impact on behavior and reproductive function. In the scope of this thesis, we focus on Endocrine disrupting chemicals (EDCs) as environmental factors able to disrupt puberty, ovulation and behavior. EDCs have a major impact on public health because of their ubiquity, complexity and implication in the onset of disease. In this chapter, we will review general concepts regarding EDCs such as their mechanisms of action, non-monotonic effects and windows of vulnerability.

3.1 Definition

Since the first definition of EDCs in 1991, the concept has substantially evolved with the help of a still growing body of scientific research (Schug et al., 2016). The World Health Organization defined the concept of EDCs in 2013 as "*an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations*" (WHO/UNEP, 2013). This definition has been adopted by the European commission (Munn and Goumenou, 2013). The European Food Safety Authority (EFSA) defines EDCs rather as endocrine active substances (EASs), "*any chemical that can interact directly or indirectly with the endocrine system, and subsequently result in adverse effects on the endocrine system, target organs and tissues*".

The increasing understanding of the mechanisms of action of EDCs and their impact on physiology and functions, others than the neuroendocrine system, has led to generate a new and independent scientific consensus statement. Published by the Endocrine Society, the panel of experts defined EDCs as...

> "*...a compound, either natural or synthetic, which, through environmental or inappropriate developmental exposures, alters the hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment*"
>
> Diamanti-Kandarakis et al., 2009

This definition includes a wide range of compounds that can be categorized by their function. A non-exhaustive list of EDCs examples and their categories can be reviewed in Table 3.1. Natural EDCs are widely present in human and animal food and include soy products such as genistein and coumestrol. Among the synthetic compounds we can find plastics and plasticizers (bisphenol A, phthalates), solvents and lubricants (polychlorinated biphenyls, polybrominated biphenyls, and dioxins), pesticides (methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane) and pharmaceutical agents (diethylstilbestrol), among others.

Category	Examples	Sources / Uses
Phtalates	DEHP, DIBP, DBP	Plasticizer, consumer products (i.e. cosmetics, dental material)
Bisphenols	BPA, BPF, BPS	PVC and epoxy resins, food containers, building material
Parabens	MeP, BP, PrP	Consumer products (i.e. cosmetics, personal care)
Pesticides / fungicide	TCS, ATZ , MTX	Agriculture runoff, vegetables
UV-Filter	4-MBC, OMC,3-BC	Sunscreens and cosmetics
Pharmaceutical	DES, ACM, EE3	Contraception, analgesic/antalgesic
Flame retardants	BDE-47, BDE-99, TBBPA	Furniture, dietary, carpet backing
PCBs	ARO1254, ARO1248	Insulating fluids, lubricanting oils, building material additives
Phytoestrogens	GEN, COU	Soybean, sesame seeds, oats
Dioxins and dibenzofurans	TCDD	Industrial by-product
Alkylphenolic compounds	PP, NP, OP	Detergents, fuel and lubrificant additives
Perfuoroalkyl compounds	PFOA, PFOS	Repellents and coating for cookware, textiles and carpets

Table 3.1. Sources of endocrine disrupting chemicals.

3.2 Mechanisms of action

The understanding of the mechanisms of action of EDCs is not complete and varies depending on the compound and its molecular structure. Although EDCs may interfere with every possible hormonal pathway, the most well-known mechanism is related to nuclear receptor binding (Diamanti-Kandarakis et al., 2009; Gore et al., 2015). EDCs are known to bind and act as agonist of antagonists of hormone receptors, such as the estrogen receptors (ER), the androgen receptor (AR), the glucocorticoid receptor (GR) and the thyroid hormone receptors (TRs) (Swedenborg et al., 2009; Shanle and Xu, 2011). By doing so, EDCs enhance, dampen or block hormonal action, in turn altering the function of hormone receptors in specific regions and organs. Additionally, EDCs can also interact with the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor (Swedenborg et al., 2009). Every compound can have different affinity for different hormone receptors and interact with them as agonist or antagonist. For example, the plasticizer Bisphenol A (BPA) is a weak ER agonist (Sengupta et al., 2013; Kuiper et al., 1998), an AR antagonist (Lee et al., 2003), has strong affinity for EERγ (Pupo et al., 2012; Matsushima et al., 2007) and is able to activate ER signaling cascades through MAPK-pErk and GPER-pErk pathways (Vinas and Watson, 2013; Ge et al., 2014; Dong, Terasaka, and Kiyama, 2011). Other EDCs, such as DDT, PFOA and Phtalates have also affinity for ERs (Kuiper et al., 1998; Miyashita et al., 2005; Xin et al., 2019; Toda et al., 2004).

EDCs can interfere with hormone biosynthesis and metabolism (Swedenborg et al., 2009). When endogenous ligands bind to ER or AhR nuclear receptors, it triggers conformational changes leading to recruitment of coactivators and to receptor dimerization with RXR or ARNT, respectively. By activating these transcription factors, gene expression is up or downregulated. EDCs can interfere with all steps of this process as for example inducing receptor degradation or interfering with coactivator recruitment (Swedenborg et al., 2009).

EDCs are able to target specific cell populations of somatic cells and interfere with the cell chromatin state by modulating transcriptional activity at specific loci of the genome (Uzumcu, Zama, and Oruc, 2012). It is still not clear whether EDCs can interact directly with proteins involved with chromatin remodeling but it seems more likely that they manipulate the epigenetic machinery by modulating expression of transcription factors dependent on nuclear hormone receptors. Importantly, EDCs are also able to specifically targeting germ cells, leading to changes in epigenetic inheritance that may contribute to the onset of disease (Crews et al., 2007; Anway and Skinner, 2008; Crews et al., 2012; Anway et al., 2005).

3.3 Non-monotonic action of EDCs and current regulatory toxicology

During the last decades, there has been increased evidence concerning the effects of EDCs defying the Paracelsus principle of "*the dose makes the poison*" (Vandenberg et al., 2012). According to this classic toxicological concept, a substance can only be harmful when exceeding a certain dose at which biological systems are susceptible. Current regulatory toxicology uses the concepts of no observable adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL) to define these thresholds. A security threshold, defined as the acceptable daily intake (ADI) is used by agencies as the U.S. Environmental Protection Agency (EPA) in USA or the EFSA in Europe, by dividing the NOAEL by a safety factor (EPA, 1993). This factor consists of multiples of 10 that add depending on the uncertainty respect to a certain topic (i.e. known differences in response between laboratory animals and humans, known susceptibility among individuals). However, as it happens with endogenous hormones, EDCs can act

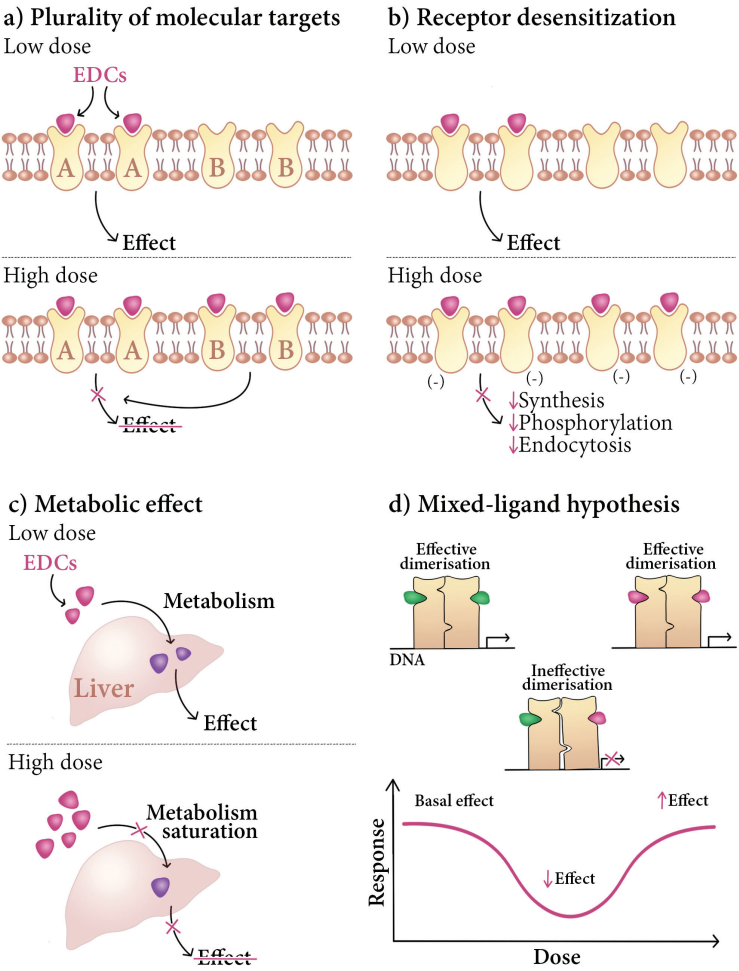


Figure 3.1. Mechanisms involved in the non monotonic dose response curves observed after EDC exposure.

at extremely low doses and induce effects that are not dose-dependent but rather fitting to a nonmonotonic dose-response curve (NMDRC) (Vandenberg et al., 2012).

NMDRC implies that "*the slope of the dose-response curve changes sign from positive to negative or vice versa at some point along the range of doses examined*" (Lagarde et al., 2015). By using this concept, defining the effects at a certain

high dose is not a helpful indication of what happens at lower doses. Multiphasic curves or U-shaped and U-inversed curves are among the NMDRC observed after exposure to endogenous hormones and EDCs. We can identify at least four different mechanisms causing a NMDR relationship (Lagarde et al., 2015).

First, EDC can interact with a plurality of molecular targets (Fig.3.1.A). At low doses, a compound may bind to a certain receptor with high affinity triggering a specific response. At higher doses, the compound can additionally bind to other receptors than may induce the opposite effect.

Second, by desensitizing receptors (Fig.3.1.B). A low dose induces the binding to a few receptors to trigger a response, whereas at higher doses numerous receptors are occupied, resulting in desensitization.

Third, by interacting with metabolisation (Fig.3.1.C). At low doses, a compound is catabolized into active metabolites inducing an effect, whereas higher doses may saturate the metabolic system inducing the opposite effect.

Fourth, by interfering with receptor dimerization (Fig.3.1.D). For a hormone to induce a response through a nuclear receptor requires the dimerization of two receptors bound to its ligand. At low doses, a compound may induce a mixed-ligand dimerization in which dimers are formed by receptors activated by different ligands. These dimers may be ineffective so transcription is blocked. However, at high doses of a compound, dimers are more likely to be formed by the binding of two receptors activated by the same ligand (a hormone or a an EDC) (Vandenberg et al., 2007).

Because of the existence of these NMDRC mechanisms, current regulatory toxicology fails to encompass real world situations in which EDCs may act at really low doses through a myriad of pathways far below any ”safe” threshold. Additionally, thresholds is often based on specific outcomes that may not apply to others. For instance, the Organisation for Economic Cooperation and Development (OECD) defined the BPA threshold by changes in uterine weight, whereas lower doses are known to affect other tissues such as the hypothalamus

(Rebuli et al., 2014; Delclos et al., 2014).

3.4 The importance of mixtures of EDCs

In real-world situation, we are not exposed to a single compound but to complex mixtures from different environmental sources. The ubiquity of EDCs in the environment and consumer products makes humans daily exposed to hundreds, of compounds that can accumulate in tissue of the body. For instance, a French nation-wide program documented the presence of EDCs among pregnant women. In the cohort of 4,145 individuals, the presence of BPA, phtalates, pesticides, dioxins, furans, PCBs, brominated flame retardants, perfluorinated compounds and heavy metals was found in virtually all pregnant women (Dereumeaux et al., 2016). This study, far from being anecdotal (Woodruff, Zota, and Schwartz, 2011; Rosofsky et al., 2017; Lee et al., 2017; Philips et al., 2018), demonstrates the importance of studying EDC mixtures.

The impact of EDC mixtures on human health-related outcomes are illustrated in several recent studies. A recent study by Tanner et al., 2020 found that prenatal exposure to 26 EDCs is correlated with lower IQ in children at 7 years old. Others studies have shown an association of EDC mixture exposure to neurodevelopmental disorders such as internalizing and externalizing behaviors (Furlong et al., 2017; Valeri et al., 2017), and female and male reproductive alterations, such as polycystic ovary syndrome (PCOS) (Yang et al., 2015; Vagi et al., 2014) and decreased semen quality (Hauser et al., 2006; Richthoff et al., 2003; Abdelouahab, Ainmelk, and Takser, 2011; Meeker et al., 2010), among others.

Despite of the exposure to multiple compounds and the evidence of health-related outcomes, at least 95% of the current toxicological research focuses exclusively on single compounds (Kortenkamp, 2007).The last few years, experts have underlined the importance of studying EDC mixtures in a regulatory context (EFSA, 2019). Still, the risk assessment of EDC mixtures is a complex topic subjected to debate. The existence of different possible approaches, the lack of theoretical concepts in mixture toxicology and the methodological difficulties make it challenging to proceed experimentally. We cite here some of the important limitations and concerns when studying EDC mixtures:

1. It is uncertain whether mixtures of compounds can act in an additive, antagonist or synergistic fashion. Additive or antagonistic effects imply that the chemicals in the mixture act together or act in contraposition to each other, respectively. A synergistic mechanism implies that the effects are greater than what we would expect by the simple addition of all compounds. Still, additive effects are difficult to establish. Alternatively, some authors have used the additivity concept based on the dose addition of compounds having similar mechanisms of action. This means that we would more easily determine the expected additive effects of a mixture when all compounds act through the same pathway. Research in the topic has focused on studying mixtures of estrogenic, antiandrogenic and thyroid-disrupting chemicals (Kortenkamp et al., 2007). This approach does not take into account the multiplicity of receptors by which a single compound can interact with. Nevertheless, it allows to demonstrate that additive and synergistic effects are a reality in EDC mixtures, that may be more harmful as a mixture than individually. This information is crutial for regulatory purposes (Kortenkamp, 2007).

2. Epidemiological data shows that different EDCs and levels of exposure are found in humans from different sub-populations. In addition, *in vivo* or *in vitro* studies demonstrating the endocrine activity of chemicals to which humans are exposed are not always available. This diversity in exposure represents a challenge to design experiments that represent to real world situations.

3. Several categories of EDCs are known to be persistent as they bioaccumulate in adipose tissue throughout their lifespan. Based on this observation, the daily dose of each compound in a mixture that contains persistent chemicals is not the only one that must be considered (Ribeiro, Ladeira, and Viegas, 2017).

4. For practical reasons, most of EDC mixture do not study the effect of each components individually. This approach is extremely useful when trying to decipher the effects and mechanisms of the compounds present in a specific population. It represents a real world approach comparable to epidemio-

logical data. The caveat arises from the difficulty to extrapolate to other mixtures, as small differences in the mixture can lead to differences in the observed effects of the compound.

3.5 Windows of exposure and vulnerability to EDCs

Exposure to EDCs represents a bigger concern during development than during adulthood (Diamanti-Kandarakis et al., 2009). EDCs target mainly parts of the endocrine system, which plays a critical role in development, especially during fetal life when hormones determine sexual differentiation. The classical view of brain sexual differentiation in mammals holds that sex differences mostly depend on the production of testosterone arising from the differentiation of the gonads into testes. Some of the effects of testosterone result from the action of estradiol derived from local aromatization of testosterone in the male brain. More recently, studies using aromatase knockout mice (Bakker et al., 2002; Brock, Baum, and Bakker, 2011) have shown that estradiol is required for female brain development as well. In rodents, the programming of male typical neural and behavioral characteristics takes place around G14 and extends to P10, whereas female-typical neural and behavioral characteristics seem to develop later, starting around P15, perhaps extending into puberty (Brock and Bakker, 2011). In humans, the sexual differentiation of the brain is thought to occur between 8-24 weeks of gestation when testosterone levels are higher in male than female fetuses (Reyes et al., 1974; Nagamani et al., 1979; Hines et al., 2002; Finegan, Bartleman, and Wong, 1989).

Those periods define windows of sensitivity for potential action of EDCs, at which the organism is more vulnerable. During these periods, EDC induce organizational changes in the endocrine system causing long-term consequences (Diamanti-Kandarakis et al., 2009). This long-lasting effects of EDC fit into the well-described concept of the developmental origin of health and disease (DoHaD). This concept is also called Barker hypothesis as he demonstrated that *in utero* exposure to undernutrition may lead to adult metabolic and cardiovascular diseases (Barker, 1995).

During early development, the organism is not only more vulnerable to environmental factors but the concentrations of EDCs are higher than adulthood (Vandenberg et al., 2007). EDCs can be transferred through the transplacental

barrier from the mother to the fetus (Barr, Bishop, and Needham, 2007). While persistent organic compounds can be slightly retained, most of other compounds such as polybrominated diphenyl ethers (PBDEs) and BPA cross the placenta, reaching the fetus (Li et al., 2013). Finally, lactation is another important source of mother-infant transmission of EDCs (Stefanidou and Spiliopoulou, 2009).

3.6 The concept of transgenerational inheritance

Early intrauterine exposure to EDCs leads to long lasting epigenetic and phenotypic changes, including the risk of disease, throughout generations (Brehm and Flaws, 2019). This transgenerational inheritance is defined as the transmission of a biological trait to the subsequent generations through the germ line without direct exposure (Fig.3.2). The exposure route for transgenerational effects for males and females differs. In an exposed gestating F0 female, the F1 em-

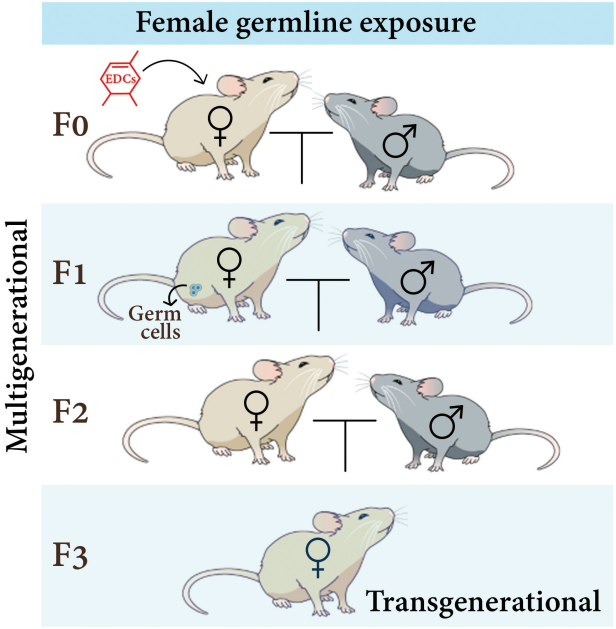


Figure 3.2. Schematic representation of multigenerational and transgenerational effects of EDCs through the female germline.

bryo and F2 generation germ line are exposed (multigenerational inheritance), whereas the F3 and subsequent generations are not directly exposed (transgenerational inheritance). In males, when the F0 is exposed, the F1 exposed through the germline is considered multigenerational and F2 is the first generation not directly exposed (transgenerational inheritance). Although there is a lack of information on how embryonic programming affects adult phenotypes, epigenetic transmission through alterations in the germline is most likely the mechanism by which the genome gains flexibility when under environmental pressures.

Since the first seminal study of Skinner in 2005, it has been established that EDC effects can be transmitted through the germline (Anway et al., 2005). Importantly, these germline-induced effects caused by EDC exposure appear to alter somatic cell development of multiple organs and tissues and specifically target the brain. For instance, exposure to vinclozolin (Crews et al., 2007; Anway and Skinner, 2008; Crews et al., 2012) affects mate preference or stress response and alters mRNA expression of methyl binding domain 2 (*Mbd2*) or DNA methyl transferase 3b (*Dnmt3b*) in the rat central amygdala, two genes associated with DNA methylation, in the F3 generation (Anway et al., 2005; Gillette et al., 2015). *In utero* BPA exposure also produces transgenerational effects on social recognition and activity (Wolstenholme et al., 2012) in mice, in part due to transgenerationally increased number of ERα expressing cells in the female AVPV, potentially through alterations in germ cell DNA methylation (Goldsby, Wolstenholme, and Rissman, 2017).

4 Endocrine disruption of the hypothalamic control of puberty and ovulation

Contents

A secular trend in pubertal timing and decreased fertility has been reported in Western countries for a few decades. Exposure to environmental pollutants such as EDCs during critical periods of development can result in delayed/advanced puberty and long-term reproductive consequences. Abnormal pubertal activation caused by EDCs may result from alterations taking place at the level of the hypothalamic GnRH network, the pituitary gland or the gonads themselves. In this chapter we will summarize the effects of EDCs on the hypothalamic control of puberty and reproduction focusing on the GnRH network throughout development, from early fetal development to adulthood.

4.1 Introduction

Between 1890 and 1960, a reduction in age at menarche has been reported in the Western world (Tanner, 1962; Parent et al., 2003). However, this trend seems to have ceased since then in most of studied countries (Parent et al., 2003; Parent et al., 2015; Aksglaede et al., 2009; Biro et al., 2010). The programming of pubertal maturation is finely tuned by sex steroids (Goy, Bercovitch, and McBrair, 1988; Herbosa-Encarnación et al., 1997) and in consequence, highly sensitive to early exposure to environmental factors like EDCs (Parent et al., 2015). EDC exposure during the perinatal or prepubertal period has been associated with early or late pubertal onset in both boys (Den Hond et al., 2011; Grandjean et al., 2012; Guo et al., 2004) and girls (Vasiliu, Muttineni, and Karmaus, 2004; Ouyang et al., 2005; Den Hond et al., 2002; Andersen et al., 2008; Wohlfahrt-Veje et al., 2012) and later disruption of the ovarian and reproductive function as polycystic ovarian syndrome (PCOS) or early menopause (Grindler et al., 2015; Barrett and Sobolewski, 2014). In rodents, environmental toxicants affect the central control of puberty (Parent et al., 2015) and ovulation as shown for the pesticide DDT (Rasier et al., 2007; Rasier et al., 2008), the plasticizer BPA (Parent et al., 2015; Franssen et al., 2016; Ruiz-Pino et al., 2019; Nah, Park, and Gye, 2011; Monje et al., 2010; Xi et al., 2011; Fernandez et al., 2009; Veiga-Lopez et al., 2014), the polychlorinated biphenyls Aroclor 1254 (Gore et al., 2002), the phytoestrogen genistein (Bateman and Patisaul, 2008; Luszczek-Trojnar et al., 2014), the alkylbenzene p-tert-octylphenol (Herath et al., 2001), among others.

The effects of EDCs on pubertal timing are variable and depend on the compound, the dose, the route and timing of exposure and the model of study. One of the most studied EDCs is the plasticizer BPA. For instance, gestational BPA exposure leads to normal (Howdeshell et al., 1999; Murray et al., 2007; Tinwell et al., 2002) or advanced pubertal timing (Honma et al., 2002; Nikaido et al., 2004). Early neonatal BPA exposure also leads to variable results, either normal (Adewale et al., 2009; Losa-Ward et al., 2012; Nagao et al., 1999; Nikaido et al., 2005a), advanced (Adewale et al., 2009; Fernandez et al., 2009; Losa-Ward et al., 2012; Nah, Park, and Gye, 2011; Franssen et al., 2014) or delayed puberty (Franssen et al., 2014; Naulé et al., 2014). In our laboratory, Franssen et al., 2014 demonstrated

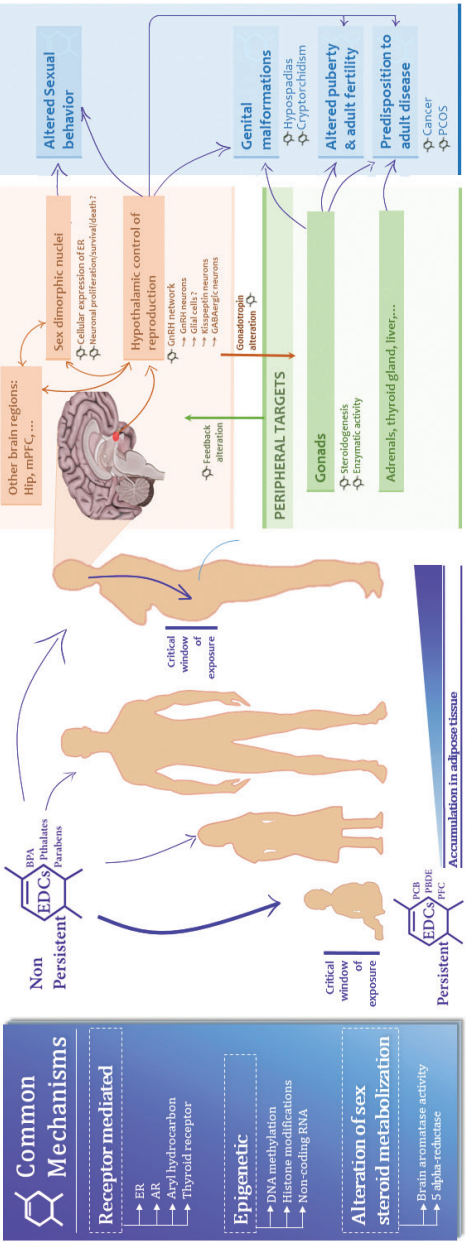


Figure 4.1. Ubiquitous presence of persistent and non-persistent Endocrine Disrupting Chemicals (EDCs) in the environment and consumer products has an impact on human health, particularly during critical windows of development. Persistent compounds are known to accumulate in adipose tissue throughout life, transfer to the fetus through the placenta and accumulate in maternal milk. Non persistent compounds are metabolized quicker but also transfer through the placenta. EDCs activate hormonal and non-hormonal receptor pathways leading to alterations in hormonal balance, epigenetic modifications and alterations of sex steroid metabolism. Such actions impact the brain and the gonads. In the brain, the development of sexual dimorphic regions and the hypothalamic control of puberty and ovulation are especially vulnerable to EDCs. Ultimately, such alterations caused by EDCs could lead to abnormal puberty, infertility and behavioral disorders and increased predisposition to adult disease.

that an early postnatal exposure to two different doses of BPA leads to opposite effects in pubertal timing. While neonatal exposure to a very low dose of BPA (25ng/kg/day) delayed pubertal timing and diminished GnRH secretion, a high dose of BPA (5mg/kg/day) advanced puberty. This study underlines in the variability of results concerning the effect of EDCs in pubertal timing, as they display a typical non-monotonic dose-response (Zoeller and Vandenberg, 2015).

The mechanisms by which EDCs alter pubertal timing may be both central or peripheral (Fig.4.1). The sensitivity of the neuroendocrine system to environmental factors (Parent et al., 2015), the ability of EDCs to cross the blood-brain barrier (Michael and Bonsall, 1990) and the role of the hypothalamus in the control of sexual maturation make it an important target of EDCs. In this context, developmental EDC exposure may disrupt the organization of the neuroendocrine reproductive axis by directly targeting the GnRH neurons or by altering transsynaptic neuron-to-neuron and glia-to-neuron inputs to GnRH neurons.

4.2 Direct effects of EDCs on GnRH neurons

Depending on the timing of the exposure to EDCs, these compounds may interfere with the differentiation or migration of GnRH neurons or disrupt their normal activity once they are mature.

Cultures of olfactory placode have provided an important means to investigate immature GnRH neurons sensitivity to steroids and EDCs. It is suspected that estrogen receptors in these neurons might play a role in sexual differentiation of the brain which takes place around that time (Wood, Greene, and Nardulli, 1998) and might be a potential target for EDCs. Acute BPA at 0.5 and 50μM, comprising the estimated circulating levels following exposure to the LOAEL (Welshons, Nagel, and Saal, 2006; Wetherill et al., 2007) inhibits intracellular calcium movement, a measure of GnRH neuronal activity in nasal placodes (Klenke, Constantin, and Wray, 2016). These effects were observed in the presence of GABA and glutamate antagonists, indicating a direct effect of BPA on GnRH neurons. This action of BPA is independent of GPR30 or EERγ and could involve voltage gated sodium channels (Wang et al., 2011; Goncalves et al., 2018). A crucial aspect of studies focusing on the *in vitro* effect of E2 or EDCs is their concentration. It is assumed that GnRH neurons are exposed to picomolar levels of E2

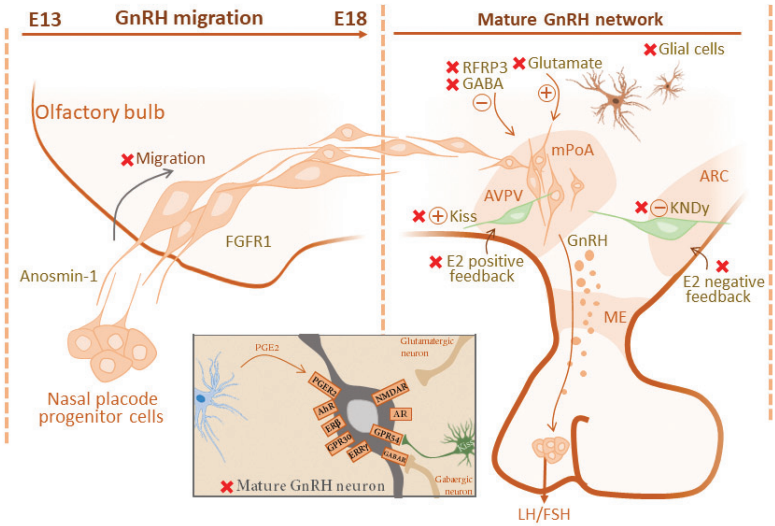


Figure 4.2. GnRH neurons originate from nasal placode progenitors cells around embryonic day 13 in the rat and migrate through the cribriform plate and across the olfactive bulb to reach the preoptic area at embryonic day 18. Migration guidance is orchestrated by olfactory axons and influenced by factors as anosmin-1 and fibroblast growth factor and prokinetic signaling. Once GnRH neurons reach the mPoA a serie of transsynaptic and glial cells conformate the GnRH neurons. Excitatory (glutamate, kisspeptin) and inhibitory (GABAergic, RFRP3) signals, regulate pulsatile GnRH secretion during puberty and ovulation. ARC Kisspeptin neurons (KNDy) play an essential role in the control of the estrogen negative feedback by interacting with the GnRH nerve terminals, whereas kisspeptin neurons of the AVPV regulate the GnRH positive feedback. GnRH neurons themselves express a wide array of receptors potentially targeted by EDCs. Known and potential targets of EDCs are marked with a red cross. RFRP3: RFamide-related peptide 3, GABA: gamma aminobutyric Acid, Kiss: kisspeptin, FGFR1: fibroblast growth factor receptor 1, AVPV: anteroventral periventricular, mPoA: median preoptic area, GnRH: gonadotropin releasing hormone, KNDy: kisspeptin, neurokinin B, and dynorphin expressing neurons, ME: median eminence, LH: luteinizing hormone, FSH: follicle stimulating hormone, PGE2: prostaglandin E2, AhR: aryl hydrocarbon receptor, ERβ: estrogen receptor beta, NMDA: n-methyl-d-aspartate, GPR54: kisspeptin receptor, ERRγ: estrogen-related receptor gamma.

(Herbison, 2009) even though such concentrations could be increased by local aromatase activity (Cornil, 2009). However, many studies have used pharmacological concentrations of estrogens (Herbison, 2009; Yewade Ng et al., 2009; Temple et al., 2004; Roy, Angelini, and Belsham, 1999), rendering them inadequate to extract accurate conclusions with physiological meaning. Thus, it is crucial to study EDC concentrations reflecting environmental exposure.

Early exposure to EDCs affects neuronal migration (Pillon et al., 2012; Bai et al., 2011) and could lead to abnormal puberty and reproduction. Oral exposure of pregnant mice to ethinylestradiol at environmental and pharmacological doses (0.01, 0.1 and 1μg/kg/day) during GnRH neuron migration (G11.5-13.5) increases the number of GnRH neurons along the migratory path without affecting their distribution (Pillon et al., 2012). In contrast, maternal exposure to 2μg/kg/d of BPA from G10 to P10 transiently decreases GnRH neuron number in pubertal male rats (Bai et al., 2011). While these studies suggest that EDCs affect the number of neurons finally reaching the preoptic area, it is unknown whether EDCs affect GnRH precursor proliferation, differentiation or migration. The potential targets of EDCs involved in GnRH migration are numerous: EDCs could directly act on GnRH neurons expressing ERβ or GPR30 early in life or affect other cell types involved in the migratory path of GnRH neurons (Kenealy, Keen, and Terasawa, 2011; Noel et al., 2009; Klenke, Constantin, and Wray, 2016).

Mature GnRH neurons convey the estrogen negative feedback onto the reproductive axis in both sexes. These cells express ERβ (Sharifi, Reuss, and Wray, 2002), GPR30 (Terasawa, Noel, and Keen, 2009), ERRγ (Klenke, Constantin, and Wray, 2016) and STX-sensitive membrane ER (Kenealy et al., 2011). While many EDCs such as BPA have relatively low affinity for ERα and ERβ (Kuiper et al., 1998), they have higher affinity for GPR30 (Terasawa, Noel, and Keen, 2009) and ERRγ (Takayanagi et al., 2006) and thus could interfere with the action of E2 on those membrane receptors. The use of immortalized GnRH neurons (i.e. mice GT1-7) provided new insights into the direct effects of EDCs on postmigratory GnRH neuron physiology, as they recapitulate their morphology and secretory capacity. However, certain limitations or differences need to be considered, like the expression of ERα by GT1-7 cells, not present in mature GnRH neurons. Using these cells, it was determined that ERα or β antagonists are unable to block the stimulatory effects of a mixture of polychlorinated biphenyl (Aroclor 1221) on GnRH transcripts and neuron morphology (Gore et al., 2002), indicating that such effects are mediated by membrane estrogen receptors or other type of non-estrogen receptors. Recent data indicates that the estrogen negative feedback results at least in part from rapid membrane-initiated E2 signaling (Herbison, 2009). However, the role of E2 membrane receptors expressed by GnRH neurons

in vivo and thus their disruption by EDCs remains to be deciphered.

Most of the studies so far have focused on EDCs that affect the estrogen pathway like BPA and DES. While these molecules certainly affect GnRH function, there is a lack of information on the effect of other widespread compounds (i.e. fungicides, herbicides and UV-filers) on GnRH neuron development and function.

4.3 Effects of EDCs on the neuronal network controlling GnRH function

As we have described in the previous chapters, once GnRH neurons are in place, pulsatile GnRH release depends on an upstream network of neurons and glial cells that provide excitatory and inhibitory transsynaptic inputs to GnRH neurons. Neurotransmitters and neuropeptides, in particular GABA, glutamate, Kisspeptins, Neurokinin B, Dynorphin and RFamide peptides play a critical role in the regulation of GnRH secretion (Terasawa et al., 2018; Zhang et al., 2009; Bourguignon, Gerard, and Franchimont, 1989). Glial cells impose a stimulatory effect to the GnRH neuronal network through secretion of prostaglandins and growth factors (Plant, 2015; Ojeda, Lomniczi, and Sandau, 2010; Prevot et al., 2003; Ojeda and Skinner, 2006). EDCs have been found to alter inhibitory and stimulatory inputs to GnRH neurons. Still today, there is a complete lack of information about EDC actions on the glial cells involved in the stimulatory control of GnRH secretion.

4.3.1 Inhibitory inputs to GnRH neurons

Although most GnRH neurons are excited by GABA, the effect of GABA is likely determined by the balance between $GABA_A$ and $GABA_B$ receptors in their soma and dendrites (Watanabe, Fukuda, and Nabekura, 2014). GABA plays a role at different developmental stages of the hypothalamic-pituitary-gonadal axis. First, it is involved in the regulation of GnRH neuron migration (Heger et al., 2003; Lee et al., 2008). Later on, a decrease in GABAergic tone is crucial for the reactivation of the GnRH machinery at puberty (Han, Abraham, and Herbison, 2002; Parent et al., 2005). GABA also appears to control the switch in estradiol feedback action in adult females (Farkas et al., 2018). Studies showed

that early EDC exposure affects GABAergic input onto GnRH neurons (Franssen et al., 2016; Cardoso et al., 2011; Cabaton et al., 2013; Zalko et al., 2016). For instance, we found opposite effects in pubertal timing caused by opposite effects in GABAergic signaling (Franssen et al., 2016). Neonatal exposure to a very low dose of BPA (25ng/kg/day) delayed pubertal maturation and diminished GnRH secretion through increased hypothalamic GABAergic neurotransmission, whereas a high dose of BPA (5mg/kg/day) advanced puberty through reduced GABAergic tone (Franssen et al., 2016). Perinatal exposure of dams to high doses of BPA in drinking water (2.5mg/kg/day) has been shown to decrease GnRH release while increasing hypothalamic GABAergic tone (Farkas et al., 2018), however, the effects on puberty onset were not studied. Although doses and periods of exposure differed, these two studies seem to indicate that EDCs such as BPA could directly affect GABAergic neurotransmission onto GnRH neurons. A study using 1H-nuclear magnetic resonance metabolomics, identified GABA as a key effector of maternal exposure (G8 to P16) to a very low dose of BPA (Cabaton et al., 2013; Zalko et al., 2016), further supporting the findings observed in the hypothalamus. Metabolic network analysis of the whole brain showed that the modulation of transaminases involved in the synthesis and consumption of L-glutamate, the primary substrate for GABA synthesis, are key mechanisms in the effects of BPA on GABAergic neurotransmission (Zalko et al., 2016). Other studies have shown that gestational exposure to mixtures of PCBs in rats alters GABA receptor expression in the POA and potentially the GnRH system receptivity to GABA (Dickerson et al., 2011). Notably, the effects varied with the nature of the PCBs and sex, indicating a potential impact on the normal development of this sexually dimorphic nucleus. In humans, sodium valproic acid exposure, a GABA agonist, was found to delay puberty in children (Lundberg et al., 1986; Cook, Bale, and Hoffman, 1992). More recently, 7 to 12 years old children living near a ferro-Manganese alloy plant in Brazil have been reported to develop early pubertal onset (Santos et al., 2019). A translational study in rats showed that exposure to manganese stimulates GnRH release and advances puberty onset decreasing GABA A receptor signalling in the preoptic area (Yang et al., 2020).

Only one publication links the effect of EDCs on RFRP3 inhibitory neuronal pathway (Ducret, Anderson, and Herbison, 2009; Kriegsfeld et al., 2010; Johnson

Target	Sex	Results	EDC	WoE	Ref
ERα mPoA	F	↑	BPA BPS Mix MTX	G G+P	Bellingham 2016 Catanese 2017 Mahoney 2010 Gore 2011
		↓	BPA 4-MBC	P G+P	Maerkel 2007 Monje 2007
	M	↓	4-MBC	G+P	Maerkel 2007
ERα AVPV	F	↑	BPA DBP	P G+P	Monje 2009 Cao 2012 Goldsby 2017
		↓	BPA PCB	P G+P	Rebuli 2014 Cao 2012 Dickerson 2011
ERα ARC	F	↑	BPA	G	Cao 2012
		↓	BPA	P	Monje 2010
	M	↑	BPA	G	Cao 2012
		↓	DEHP Mix	G	Gao 2018 Bellingham 2016
ERα VMH	F	↑	BPA	G	Cao 2012
		↓	4-MBC BPA	G+P	Maerkel 2007
	M	↑	BPA	G	Cao 2012
		↓	4-MBH	G+P	Maerkel 2007
ERβ mPoA	F	↓	BPA MTX	G G+P	Rebuli 2014 Mahoney 2010
ERβ ARC	F	↓	DEHP	G	Verbanck 2017
ERβ AVPV	F	↓	BPA PCB	P G+P	Rebuli 2014 Salama 2003 Cao 2013
	F	↑	GEN BPA	P	Patisaul 2012 Franssen 2016
	M	↑	BPA	G	Cao 2012
		↓	BPA	P	Cao 2013
Kisspeptin AVPV	F	↑	BPA PCB	G G+P	Ruiz-Pino 2019 Walker 2014 Naule 2014
		↓	BPA DES GEN	P	Franssen 2014 Losa 2011 Cao 2012 Adewale 2009
	M	↑	BPA	G+P	Bai 2011
Kisspeptin mPoA	M	↓	PCB Mix	G	Dickerson 2011 Bellingham 2016
Kisspeptin ARC	F	↓	BPA GEN	P G+P	Losa-Ward 2019 Losa-Ward 2012 Losa 2011
	M	↓	DEHP Mix	G	Gao 2018 Bellingham 2016
GABA MBH	F	Alt	PCB	P	Kumar 2017
		↑	BPA	P G+P	Yeo 2013 McCaffrey 2013
		↓	BPA	P	McCaffrey 2013
	M	↓	BPA	G+P	Guida 2014
RFRP3 MBH	F	↓	BPA	P	Verbanck 2017
Glutamate MBH	F	↑	DDT	P	Guo 2004 McCaffrey 2013
	M	↓	BPA	G+P	Vasiliu 2004
TAC2 ARC	F	↓	BPA	G+P	Ruiz-Pino 2019

Table 4.1. Cellular and molecular targets of EDCs in rodent GnRH network. WoE: Window of exposure, G: gestational exposure, P: early postnatal exposure, ↑: increased, ↓: decreased.

and Fraley, 2008) onto GnRH neurons perhaps explaining alterations in pubertal onset. Female rats exposed to 10μg/kg E2 or 50μg/kg BPA showed decreased RFRP-3 fiber density and contacts on GnRH neurons and advance pubertal development (Losa-Ward et al., 2012). Although perinatal exposure to a low dose of BPA disrupts the hypothalamic feeding circuitry by reducing NPY, POMC and AgRP projections into the ARC and the PVN (MacKay, Patterson, and Abizaid, 2017; Mackay et al., 2013), still today there is no information if some of the EDCs actions on the GnRH network is associated with disruption of this feeding circuit.

4.3.2 Excitatory inputs to GnRH neurons

Glutamate plays a crucial role in activating GnRH neurons, in particular at the time of puberty (Clarkson and Herbison, 2006a; Terasawa et al., 1999). While GnRH neurons express AMPA, NMDA and kainate receptors, the site of glutamate action within the GnRH neuronal network remains to be completely understood (Iremonger et al., 2010). Large populations of glutamatergic neurons in the hypothalamus express ERα and are involved in the transmission of the estrogen feedback (Wang et al., 2018). Studies suggest that glutamatergic neurons mediate some of the effects of EDCs on the activation of GnRH secretion around puberty (Rasier et al., 2007; Rasier et al., 2008). Neonatal exposure to the estrogenic insecticide DDT or estradiol leads to early acceleration of GnRH secretion, increased glutamate evoked GnRH release and early vaginal opening in female rats possibly trough genomic and nongenomic mechanisms (Rasier et al., 2007; Rasier et al., 2008). The rapid effects of DDT on glutamate evoked GnRH release involved ER, AhR, and AMPA receptors (Rasier et al., 2008). On the other hand, gestational and lactational exposure to a high dose of BPA (2.5mg/kg/d) decreases serum LH and testosterone associated with decreased hypothalamic release of GnRH and glutamate *in vitro* in prepubertal male rats (Cardoso et al., 2010).

The excitatory kisspeptin to GnRH neuronal contacts is fully established and operative before birth (Kumar et al., 2015). In rodents, there are two main populations of kisspeptin neurons, the ARC and the AVPV kissppeptin neurons (Smith et al., 2006). The kisspeptin neuron population in the AVPV is significantly larger in females compared to males (Cravo et al., 2011) and is involved in the preovulatory surge of LH. Seventy percent of AVPV kisspeptin neurons express ERα (Roseweir et al., 2009), making them a target for gonadal steroids (Clarkson et al., 2009) and therefore vulnerable to EDCs (Patisaul, 2013). Several studies (Ruiz-Pino et al., 2019; Navarro et al., 2009c; Franssen et al., 2014; Losa et al., 2011) indicated that kisspeptin expression around puberty is sensitive to early exposure to EDCs, but the causal link between the changes in kisspeptin expression and abnormal puberty onset remains to be elucidated. Initial studies showed that neonatal exposure to estradiol benzoate (Navarro et al., 2009c), DES (Franssen et al., 2014) or GEN (Losa et al., 2011) leads to decreased *Kiss1* expression in the hypothalamus of juvenile rats (Navarro et al., 2009c; Franssen et al., 2014) and

kisspeptin immunoreactive fibers of adult female rats (Losa et al., 2011). Very recent data suggest that the two main populations of kisspeptin neurons could have divergent sensitivity to EDC exposure (Ruiz-Pino et al., 2019). Mice exposed to low doses (G11-P8) of BPA, exhibit a persistent, but divergent, impairment of *Kiss1* neuronal maturation, with more kisspeptin cells in the AVPV but consistently fewer kisspeptin neurons and lower *Kiss1* and *Tac3* expression in the ARC (Ruiz-Pino et al., 2019). Moreover, BPA interferes with endogenous estrogens either by direct interaction with ER or by altering ERα or ERβ expression in the AVPV (Cao et al., 2014; Rebuli et al., 2014; Monje et al., 2009; Cao et al., 2012). Alterations of ERα were also observed in the ARC network controlling GnRH pulse generator (Monje et al., 2010). Together, these data suggest that developmental exposure to EDCs could distinctively disrupt the normal differentiation of both kisspeptin populations ultimately affecting pubertal timing and ovulatory capacity. Additionally, pubertal exposure to BPA through direct infusion into the median eminence in rhesus monkeys, suppresses kisspeptin secretion and subsequently GnRH release in mid- to late-puberty (Kurian et al., 2015), indicating that the pubertal period could be another window of sensitivity of the kisspeptin system to EDCs.

4.4 Effects of EDCs on estrogen positive feedback

The pubertal period appears to be another window of sensitivity for the programming of ovulation. For example, exposure to organochloride pesticides between P21-23 advances onset of puberty and alters estrous cyclicity (Laws et al., 2000) and continuous plasma infusion of BPA in prepubertal sheep reduces LH pulse frequency (Collet et al., 2010) for BPA serum concentrations about tenfold higher than human concentrations commonly documented in biomonitoring studies.

There is growing evidence of the action of EDCs through non-hormonal mechanisms, challenging the classical estrogenic/antiandrogenic vision (Everett and Sawyer, 1950; Smarr, Gile, and Iglesia, 2013; Loganathan et al., 2019; Kalil et al., 2016). For instance, clock genes in the AVPV play a key role in the control of circadian signals timing the preovulatory surge of LH (Everett and Sawyer, 1950; Smarr, Gile, and Iglesia, 2013). This mechanism seems to be independent of estrogen levels (Smarr, Gile, and Iglesia, 2013) and vulnerable to endocrine disruption.

Target	Results		EDC	WoE	Ref.
GnRH	↑	mRNA	BPA MTX	P G+P Ad	Monje 2010 Xi 2011 Gore 2011
		Secretion	4-NP BPA DDT MTX	P	Xi 2007 Franssen 2016 Franssen 2014
	↓	mRNA	BPA MTX PFOS TBT	G P Ad	Monje 2010 Mahoney 2010
		Secretion	BPA DES DPN EQ GEN OMC PPT	P	Herath 2001 Franssen 2014 Kurian 2015 Szwarcfarb 2018
Responsiveness to GnRH	↑		GEN	P	Faber 1991
	↓		ZEA	P	Faber 1991
LH surge	Altered		ATZ BP GEN MTX PFOS TBT	P G+P	Xi 2011 Veiga-Lopez 2014 Herath 2001 Feng 2015 Savabieasfahani 2008 Savabieasfahani 2006

Table 4.2. Hypothalamic and pituitary effects of EDCs on GnRH. WoE: Window of exposure, G: gestational exposure, P: early postnatal exposure, Ad: adult exposure, ↑: increased, ↓: decreased.

In vitro exposure of immortalized hypothalamic neurons to BPA alters Bmal1 and Per2 mRNA expression (Loganathan et al., 2019). While the non-estrogenic regulation of AVPV kisspeptin secretion through the action on circadian genes seems to be mediated by noradrenaline signaling (Kalil et al., 2016), there is not enough *in vivo* information linking BPA with this mechanism.

4.5 Effects of EDC mixtures in the reproductive axis

While most studies so far, have focused on single compounds, some more recent data have emerged regarding EDC mixture and reproduction. Most EDC mixture studies focused on studying estrogenic, antiandrogenic or thyroid-disrupting compounds separately (Kortenkamp et al., 2007; Lee, 2018). Perinatal exposure to a mixture of 13 compounds has been shown to alter the female reproductive system. This mixture has been extensively studied by the division of toxicology and risk assessment at the National Food Institute in Denmark and it is composed of antiandrogenic (DBP, DEHP, VIN, procymidone, prochloraz, linuron, epoxiconazole, p,p'-DDE) and estrogenic (4-MBC, OMC, BPA, BP and paracetamol) compounds. The action of every category of compounds has been studied separately or together as a complex mixture and using dose levels 200 to 450 times higher than the human exposure range (Axelstad et al., 2014). For instance, a perinatal antiandrogen EDC mixture reduces primordial follicles in prepubertal rats, alters estrous cyclicity and alters mammary gland development by reducing prolactin levels (Johansson et al., 2016; Isling et al., 2014). When estrogenic and antiandrogenic compounds are added together to the mix, the effects in estrous cyclicity were found to be more severe (Johansson et al., 2016; Isling et al., 2014).

No differences were found in pubertal timing in these studies (Isling et al., 2014).

Only a few studies using EDC mixtures have focused on deciphering the mechanisms explaining reproductive outcomes. For instance, exposure to the mixture of 13 EDCs including androgenic and estrogenic substances was found to alter expression of genes related to glutamatergic and GABAergic signaling in the mPoA (Lichtensteiger et al., 2015). Exposure to a low doses mixture of six pesticides decreases birth weight in rats (Hass et al., 2017). Using the same mixture, *Kiss1* mRNA expression in the AVPV showed no differences but they found alterations of the kisspeptin system when using the pesticide mancozeb alone (Overgaard et al., 2013). Studies of single compounds found in the mixtures described here help to understand the mechanism of action of these compounds. Specifically, BPA is known to cause oocyte nest breakdown (Wang, Hafner, and Flaws, 2014) and that may alter ovarian follicle development by increasing follicle atresia (Peretz, Craig, and Flaws, 2012). BPA was found to increase the proapoptotic factor BCL2-associated X protein (*Bax*), B cell lymphoma 2 (*Bcl2*) and transformation-related protein 53 (*Trp53*), which in turn, induce cell division arrest and DNA damage (Xu et al., 2002). Overexpression of *Esr1* in BPA treated females, did not decrease follicle atresia, suggesting that the effects are independent of this receptor. The antiandrogenic compounds DEHP and DBP accelerate primordial follicle recruitment by overactivating the PI3K pathway (Hannon and Flaws, 2015). Moreover, DBP inhibits growth of antral follicle by suppressing the expression of cyclin-dependent kinase inhibitors (Craig et al., 2013).

The objective of these studies was to define a framework for conducting cumulative risk assessment of the reproductive hazard of EDC mixtures from a toxicological perspective. The use of toxicological doses and gavage as the route of exposure make it hard to make conclusions applicable to real world situations. While gavage is still the reference for EDC studies and demanded by regulatory agencies, it has been demonstrated to induce stress responses by the endocrine system, a possible confounding factor for the risk assessment of EDCs (Vandenberg et al., 2014b; Cao et al., 2012). To the best of our knowledge, no studies have addressed the effect of EDC mixtures at low environmental relevant doses on the female reproductive system.

4.6 Effects of EDCs on the epigenetic control of the GnRH network

Epigenetic control of gene expression is very well positioned as a relay of environmental information to the gene networks controlling physiological processes (Vazquez et al., 2018), including the GnRH network (Aylwin et al., 2019). More importantly, epigenetic changes not only produce long term effects in somatic cells, but also affect the germline, inducing alterations that are inherited transgenerationally (Anway et al., 2005; Skinner et al., 2008; Crews et al., 2007; Wolstenholme et al., 2012).

4.6.1 Epigenetic changes in somatic cells

During the last several years, a significant number of studies shed new light into how epigenetic processes affect sexual differentiation (Nugent et al., 2015; Matsuda et al., 2011) and the onset of puberty (Toro et al., 2018; Lomniczi et al., 2015). Changes in DNA methylation and histone PTMs participate in brain sexual differentiation (Forger, Strahan, and Castillo-Ruiz, 2016; McCarthy and Nugent, 2013). In female rodents, the sexual dimorphic region of the preoptic area is actively repressed by DNA methylation (Nugent et al., 2015). Such repression is reversed by E2 stimulation or pharmacological inhibition of DNMTs, inducing a male phenotype (Nugent et al., 2015). On the other hand, histone deacetylation is associated with brain masculinization because pharmacological inhibition of histone deacetylase activity reduces sexual behavior in males (Matsuda et al., 2011). These results suggest that hypothalamic masculinization is an ERα dependent epigenetic phenomenon.

Important evidence was recently obtained on the crucial role that DNA methylation, histone PTMs and ncRNAs have in the regulation of the transcriptional machinery of neurons involved in reactivating the GnRH pulse generator around puberty (Toro et al., 2018; Tomikawa et al., 2012; Lomniczi, Aylwin, and Vigh-Conrad, 2019). As described before, the activation of the ARC kisspeptin neuronal population is critical for pubertal onset. In the rat ARC, the 5' regulatory region of the *Kiss1* gene contains both repressing and activating epigenetic regulators. This particular chromatin landscape allows for rapid activation and/or repression of gene expression. Before puberty, the *Kiss1* promoter is enriched in

	Target	Effect	EDC	WoE	Sex	Region	Specie	Age	Ref.
DNA Methylation									
Enzymes	*Dnmt1*	Alt	BPA	G	F M	PFC & MBH	Mice	P28	Kundakovic 2013
		↑	BPA	G+P	M	CTX & HIP	Mice	P21	Kumar 2017
		↑	BPA	G+P	F	AMY	Rat	P45	Zhou 206
		↑	BPA	G+P	M F	CTX	Mice	Adult	Malloy 2019
		↓	BPA	G+P	M	CTX & HIP	Mice	Adult	Kumar 2017
		Alt	A1221	G	F	AVPV	Mice	P28	Walker 2014
	Dnmt3a	Alt	BPA	G	F M	PFC & MBH	Mice	P28	Kundakovic 2013
		↑	BPA	G+P	M	CTX	Mice	P21	Kumar 2017
	Dnmt3b	↓	BPA	G+P	M	MBH	Rat	Adult	Kaelin 2013
		↓	Vin	G	F	VMN	Rat	F3 Adult	Gillette 2015
	5-mC	↓	BPA	G+P	M	CTX & HIP	Mice	P21 Adult	Kumar 2017
Methylation	*Esr1*	↓	EB	G+P	F	MBH	Rat	Adult	Gore 2011
		↑	BPA	G	M	PFC	Mice	P28	Kundakovic 2013
		↑	BPA	G	F	MBH	Mice	P28	Kundakovic 2013
	Bdnf	↑	BPA	G+P	F	HIP	rat	Adult	Cheong 2018
	Mbd2	↑	VIN	G	F	CeA	Rat	F3 Adult	Gillette 2015
	Grin2b	↓	BPA	G+P	F	HIP	Rat	Adult	Alavian-Ghavanini 2018
Non-coding RNA									
lncRNA	*Meg3*	↑	BPA	G	M	MBH	Mice	F3 P28	Drobna 2018
miRNA	*mir-219, let-7a*	↑	A1221	G	F	mPoA	Rat	P30	Topper 2015
	mir-219, let-7a	↓	A1221	G	M	mPoA	Rat	Adult	Topper 2015
Histone PTMs									
Enzymes	*Hdac2*	↑	BPA	G+P	M	CTX	Mice	P21 Adult	Kumar 2017
		↑	BPA	G+P	M	HIP	Mice	P21	Kumar 2017
		↓	BPA	G+P	M	HIP	Mice	Adult	Kumar 2017
Histone PTMs	*H3K9ac*	↑	BPA	G+P	M	CTX & HIP	Mice	P21 Adult	Kumar 2017
	H3K14ac	↑	BPA	G+P	M	CTX & HIP	Mice	P21 Adult	Kumar 2017

Table 4.3. Effects of EDCs on DNA methylation, non-coding RNA and histone posttranslational modifications (PTMs) in the brain. WoE: Window of exposure lncRNA: long non coding RNA, miRNA: micro RNA, ↑: increased., ↓: decreased. G: gestational, P: postnatal.

the repressing histone H3 trimethylated at lysine 27 (H3K27me3), induced by the presence of the Polycomb Group (PcG) of epigenetic silencers (Lomniczi et al., 2013b). As puberty approaches the Trithorax Group (TrxG) of epigenetic activators, counteracts these repressive marks by imposing activational H3K4me3 and H3K27Ac PTMs at the ARC *Kiss1* promoter and enhancer regions respectively (Toro et al., 2018). After completion of puberty, the ovary dependent activation of the AVPV kisspeptin neurons during the preovulatory surge of LH requires increased histone H3 acetylation at the *Kiss1* promoter/enhancer region in an estrogen dependent manner (Tomikawa et al., 2012). Moreover, the H3K27 demethylase JMJD3 has been shown to control female puberty and ovulation by regulating AVPV *Kiss1* gene expression in an E2 dependent fashion (Song et al., 2017).

EDC exposure during development could affect DNA methylation, histone PTM or ncRNAs expression (Fig.4.3). Initial studies looking at the epigenetic effects of EDCs in the brain identified changes in DNMT expression (Gillette and Hill, 2015; Anway et al., 2005; Walker, Goetz, and Gore, 2014; Desaulniers et al., 2005) or methylation changes at specific loci (Cheong et al., 2018). *In utero* BPA exposure results in sex-specific and dose-dependent disruption of DNMTs expression in the young (P28) and adult hypothalamus (Cheong et al., 2018; Kundakovic et al., 2013), paralleling changes in mRNA expression of the estrogen receptors *Esr1*, *Esr2* and *Esrrg* in the juvenile mouse brain (Kundakovic et al., 2013). Such exposure abolishes sex differences in social, exploratory and anxiety-like behavior in part explained by changes in ER expression. Maternal care is able to attenuate these effects, suggesting that postnatal environment modulates alterations in epigenetic programming induced by EDCs. While most of these studies identify changes in mRNA expression of key enzymes involved in CpG methylation or histone PTMs, EDCs could also affect the levels of co-factors involved in their activity, especially SAM. Although, there is still a complete lack of information on how EDCs affect cellular levels of substrates and co-factors involved in the enzymatic epigenetic processes at the GnRH network, much needs to be learned from studies on other models. It is known that SAM is a methyl donor for practically all methylation reactions within a cell. SAM is produced from dietary methionine by a methionine adenosyltransferase (MAT), depletion of SAM leads to a global reduction in methylation capacity (Kaelin and McKnight, 2013). *In vivo* exposure to 3-methyocholanthrene,a polycyclic arylhydrocarbon, reduces liver *Mat* mRNA and protein levels (Carretero et al., 2001) in adult rats, while BPA exposure in the agouti mouse model induces DNA hypomethylation through SAM depletion, and is reversed by diet supplementation with folic acid (Dolinoy, Huang, and Jirtle, 2007). While histone methylation is dependent on the cellular levels of SAM and flavin adenine dinucleotide (FAD), a co-factor of some lysine demethylases, histone acetylation is dependent on cellular levels of Acetyl-CoA (a co-factor for histone acetyl transferases) and nicotinamide adenine dinucleotide (NAD+), a co-factor for the Sirtuin family of deacetylases (Kaelin and McKnight, 2013).

While the epigenetic control of ovulation appears to be sensitive to environ-

mental factors such as nutrition (Vazquez et al., 2018), no study so far has explored the effects of EDCs on these epigenetic mechanisms. However, EDCs can alter estrous cyclicity and accelerate reproductive senescence through life-long changes in hypothalamic gene expression (Walker, Goetz, and Gore, 2014; Armenti et al., 2008; Adewale et al., 2009; Shi et al., 2007). In particular, methylation of key hypothalamic genes like *Esr1* is found altered in 16 month-old female rats after gestational and neonatal exposure to estradiol benzoate (Gore et al., 2011), an exposure that leads to premature ovarian senescence.

Very few studies have reported *in vivo* effects of EDCs on histone PTMs in the brain. BPA and phthalates have been reported to alter chromatin structure in primary cortical neuron cultures (Yeo et al., 2013), neuroblastoma cell lines (Guida et al., 2014) and other tissues (Seachrist et al., 2016). More recently, perinatal BPA exposure has been shown to persistently increase histone H3 acetylation in the cerebral cortex and hippocampus of postnatal male mice (Kumar and Thakur, 2017). However, no study so far has reported effects of EDCs on histone PTMs in the neuronal networks controlling GnRH release.

It is known that miRNA expression in the mPoA is sexually dimorphic and sensitive to perinatal estrogens (Topper, Walker, and Gore, 2015). BPA affects miRNA expression in the ovary (Veiga-Lopez et al., 2013), testis (Gao et al., 2018) and adipocytes (Verbanck et al., 2017) and some miRNAs and their transcriptomic responses appear significantly associated with the presence of persistent organic pollutants in the blood of healthy subjects (Krauskopf et al., 2017). Only one study has shown that a short gestational exposure to a PCB mixture leads to an increase in the expression of several miRNA (mir-219, mir-132, mir-7, mir-145, let-7a) in the female mPoA around puberty but a decreased expression in miRNAs in the male hypothalamus at adulthood (Topper, Walker, and Gore, 2015). However, few target genes were affected with such exposure. The similarities between the effects of the PCB mixture and estradiol benzoate in females suggest that the mechanisms that underlie this increase in miRNA expression is of estrogenic nature.

While most of the aforementioned epigenetic effects of EDCs are associated with their classical agonist or antagonist action on sex steroid receptors and thus, gene expression, more research needs to be done to understand how fluctuations

EDC	WoE	Transgenerational effects	Ref.
Female			
BPA	G	Altered imprinted genes in the PoA	Wolstenholme 2012
DEHP		Altered folliculogenesis	Pocar 2017
		Reduced ovarian primordial follicular reserve	Pocar 2017
TCDD		Altered puberty	Manikkam 2012
		Reduced ovarian primordial follicular reserve	Manikkam 2012
DEET		Altered puberty	Manikkam 2012b Manikkam 2012c
		Reduced ovarian primordial follicular reserve	Manikkam 2012b Manikkam 2012c
EDC Mixture		Altered puberty	Manikkam 2013
		Obesity	Manikkam 2013
		Reduced ovarian primordial follicular reserve	Manikkam 2013 Nilsson 2012 Tracey 2013
		Altered DNA methylation in ovaries	Nilsson 2012
Male			
VIN		Decreased fertility / spermatogenesis	Anway 2005 Anway 2006 Anway 2008
		Prostate disease	Anway 2008
		Altered mate preference	Crews 2007
		Increased anxiety-like behavior	Skinner 2008
		Altered stress responsiveness	Crews 2012
		Altered DNA methylation in testis/sperm	Anway 2005 Guerrero-Bosagna 2010 Guerrero-Bosagna 2013 Skinner 2013
		Altered Dnmt mRNA in sperm	Anway 2008
		Sexual dimorphic alteration of brain transcriptomics	Crews 2007 Skinner 2008 Gillette 2014
		Altered ncRNA in PGCs	Brieno-Enriquez 2015
		No effects	Schneider 2008 Stouder 2010 Inawaka 2009 Schneider 2013 Iqbal 2015
MTX		Altered imprinted genes / methylation in sperm	Stouder 2011 Manikkam 2014
DDT		Altered DNA methylation in sperm	Skinner 2013
BPA	G+P	Altered Esr1 mRNA in the brain	Wolstenholme 2012
		Impaired dishabituation to a novel female	Wolstenholme 2013
		No effects	Iqbal 2015
DEHP	G	Decreased fertility / spermatogenesis	Doyle 2013
		Altered Dnmt mRNA in sperm	Chen 2015
TCDD		Altered DNA methylation in sperm	Manikkam 2012
EDC Mixture		Altered DNA methylation in sperm	Tracey 2013

Table 4.4. Transgenerational effects of EDCs on male and female reproduction and metabolism. WoE: Window of exposure G: gestational, P: postnatal

in cellular metabolites affect the epigenetic machinery in the GnRH network and how different EDCs interfere with this process.

4.6.2 Epigenetic changes in germ cells

Multi- and transgenerational studies have provided evidence of the persistent effects of EDCs on the male and female reproductive axis (Steinberg et al., 2008; Crews et al., 2007; Anway and Skinner, 2008; Crews et al., 2012). Gestational exposure to Aroclor 1221 suppressed LH and progesterone levels in rats exposed while *in utero*, a phenotype more severe in the offspring, suggesting that alterations in the hypothalamic gene network is transmitted to F2 animals (Steinberg et al., 2008). EDC effects appear to be transmitted through the germline (Fig.4.4). For instance, exposure to vinclozolin (Crews et al., 2007; Anway and

Skinner, 2008; Crews et al., 2012) affects mate preference or stress response and alters mRNA expression of *Mbd2* or *Dnmt3b* in the rat central amygdala, two genes associated with DNA methylation, in the F3 generation (Anway et al., 2005; Gillette and Hill, 2015). *In utero* BPA exposure also produces transgenerational effects on social recognition and activity (Wolstenholme et al., 2012) in mice, in part due to transgenerationally increased number of ERα expressing cells in the female AVPV, potentially through alterations in germ cell DNA methylation (Goldsby, Wolstenholme, and Rissman, 2017). Other studies have demonstrated the presence of transgenerational alterations in DNA methylation, *Dnmt1* and ncRNA expression in the male germline after exposure to vinclozolin (Anway et al., 2005; Guerrero-Bosagna et al., 2010; Guerrero-Bosagna et al., 2013; Anway and Skinner, 2008; Brieno-Enriquez et al., 2015), Methoxyclor (Stouder and Paoloni-Giacobino, 2011; Manikkam et al., 2014), DDT (Skinner et al., 2013), DEHP (Doyle et al., 2013) and TCDD (Manikkam et al., 2012a)). However, the effect of EDCs on the female germline is less well known.

EDC exposure also leads to transgenerational effects on pubertal timing and ovarian function (Nilsson et al., 2012; Tracey et al., 2013; Pocar et al., 2017; Manikkam et al., 2012a; Manikkam et al., 2012b; Manikkam et al., 2013), but the epigenetic pathways conveying information from a wide range of stimuli to the hypothalamic neurons regulating the onset of puberty still need to be elucidated. Hypothalamic long ncRNAs play a role in the control of puberty and reproduction (Gao et al., 2017) and could be transgenerationally targeted by perinatal exposure to BPA (Drobna et al., 2018). Among these genes, maternally expressed gene 3 (*Meg3*), an imprinted lncRNA associated with precocious puberty (Geoffron et al., 2018) and neurobehavioral problems (Fuemmeler et al., 2016), is increased in the mPoA of F3 juvenile males exposed to BPA. However, DNA methylation of this gene was not affected by BPA (Drobna et al., 2018), suggesting that other epigenetic mechanisms, such as histone PTMs, could explain the transgenerational alteration of Meg3 expression.

Although expensive and time consuming, transgenerational studies are needed to identify the effect of different EDCs on the GnRH network in order to understand how life-long exposure to low doses of EDCs or EDC mixtures affects reproductive development and fertility throughout generations, especially

in view of the secular trend of timing of puberty (Parent et al., 2015) and fertility (Minguez-Alarcon et al., 2018) in humans.

5 Maternal behavior and endocrine disruption

Contents

Maternal care is an ensemble of incredibly motivated behaviors, essential for the development of the offspring. From the mothers perspective, the display of maternal care at delivery depends on hormonal levels during pregnancy that activate the maternal brain network. Postnatally, sensorial cues allow its continuity. From the pups perspective, mothering stimulates and shapes the offspring's brain. Maternally-induced organizational changes in the brain have long-lasting consequences and may be transmitted throughout generations. For instance, individual variability in maternal care is transmitted to the next generation. Thus, maternal care can serve as the basis for a non-genomic multigenerational inheritance. In this chapter, we will describe the role of the mPoA in orchestrating maternal care and the little evidence on the effect of EDCs on this behavior. This region integrates sensorial and hormonal cues to inhibit pup avoidance and activate promaternal behaviors.

5.1 Components of maternal behavior

In nonhuman mammals we can define maternal behavior as "*a fairly stereotyped constellation of behaviors on the part of the mother wholse function is to prepare her for the arrival of the newborn, take care of it, and eventually, promote the independence of the offspring*" (Kristal, 2009). Rats have been extensively used as a model for the study of maternal care because they are altricial species, as humans, and because they display a highly stereotyped and complex constellation of behaviors easily quantified in the laboratory (Dollinger, Holloway, and Denenberg, 1980; Kristal, 2009).

The onset of promaternal behaviors starts before birth and increases in complexity and frequency throughout pregnancy. Rats spend increasingly and progressively more time grooming and are able to create high-walled nests, as a consequence of the decrease of body temperature throughout pregnancy (Kristal, 2009; Lonstein et al., 2015; Hennessy et al., 1980). At birth, dams lick the entire body of the altricial newborns and retrieve them to the nest. At this time, dams display periodic intervals of promaternal behaviors that depend on hormonal and non-hormonal factors as well as sensorial inputs received from the dyadic interactions with the pups. Pup ultrasonic vocalizations and their odors attract the mother and promote the beginning of maternal care (White and Barfield, 1987). This is, nursing, licking, grooming, mouthing and retrieval. Low frequency ultrasonic vocalizations (around 22-23 kHz) trigger nursing, which is the display of a position in which pups are able to suckle (Hennessy et al., 1980; Stern and Lonstein, 2001). In rats, dams nurse under the kyphosis or arched-back position, in which they display a dorsal flexion of the spine and extended legs (Stern and Lonstein, 2001; Altemus et al., 2004). Alternatively, a passive and blanket position have also being observed, in which females lay down on the side allowing pups to suckle. Generally, nursing behavior is considered as a physiological process rather than a behavior, in which lactation is triggered (Jesseau, Holmes, and Lee, 2008; Wakerley, 2006). High frequency ultrasonic vocalizations (40-50 kHz) trigger the retrieval of pups to the nest (Brunelli, Shair, and Hofer, 1994). During this behavior, mothers grip the pup's back returning them to the nest. Finally, licking and grooming (LG) pups is a key component of maternal care as it

is highly variable among dams and determine different aspects of offspring development (Curley and Champagne, 2016). Once pups are satiated and they slow down their demands, dams may leave the nest to self-grooming or rest until the next sequence of maternal care is required.

The display of maternal behavior evolves throughout the postnatal period until weaning at postnatal day (PND) 21, when pups are fully independent. Nursing behavior becomes less necessary as pups become independent to eat and retrieval increases over time as youngs become motorically more mature (Reisbick, Rosenblatt, and Mayer, 1975). For instance, at the second week of age pups do not require to display vocalizations to attract the mother as they are independent enought to initiate feeding bouts by themselves. By the end of lactation and afterwards, dams start avoiding the contact with pups as they may be a source of pain caused by the growing teeth and the inability to accomplish their demands (Cruz et al., 1996).

5.2 Hormonal control of maternal care

The hormonal changes in estradiol, progesterone, prolactin and oxytocin during pregnancy are crucial for the rapid onset of maternal care after delivery (Fig.5.1.A). Rodents are known to display a natural avoidance toward pups and virgin females requires a continuous exposure to pups to activate parenting. This artificial sensitization of maternal care can activate the whole constellation of maternal behaviors but they display lower record of times spent in retrieval, pup licking and nursing (Bridges et al., 1972; Lonstein, Wagner, and De Vries, 1999; Champagne et al., 2001). In addition, hormonal treatment at levels compared to those found in late pregnancy to virgin females triggers a rapid onset of maternal care (Moltz et al., 1970; Rosenblatt and Ceus, 1998). These results clearly demonstrate the importance of pregnancy events to induce mothering. During the postportum period, hormonal levels become progressively less important. The maintenance of the behavior seems regulated by the dyadic interactions between mother and infant (Fig.5.1.B). This was demonstrated by early studies showing that ablation of the pituitary, ovary or adrenal glands after delivery does not produce a major impairment of maternal care (reviewed in Numan and Insel, 2003).

In rodents, during pregnancy, estradiol levels remain low until G16, when

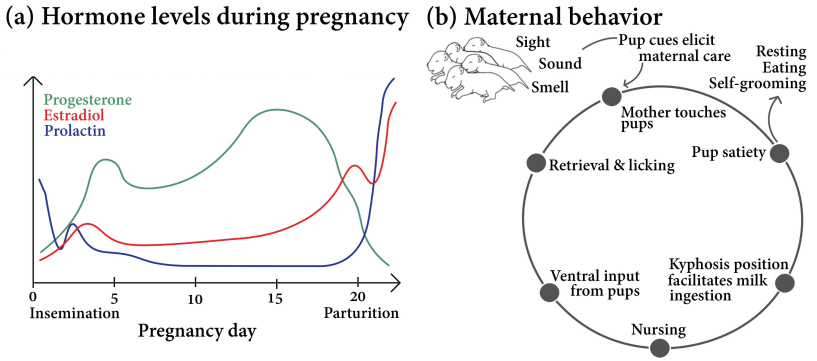


Figure 5.1. Hormonal changes during pregnancy and components of maternal behavior. (A) Schematic representation of changes in estradiol, progesterone, and prolactin during pregnancy. Role of sensorial cues regulating maternal behavior. In this model, pup ultrasonic vocalizations and smell elicit the beginning of a periodic sequence of maternal care. This sequence includes retrieval, licking and nursing. Pup satiation reduces the sensorial input and mother may depart until another sequence of mothering is required.

there is a peak that reaches superior levels to those found during the estrous cycle (Bridges, 1984; Shaikh, 1971; Garland et al., 1987). After delivery, there is a short surge causing receptivity, followed by low levels that progressively rise throughout the lactational period ultimately triggering the recovery of the estrous cyclicity (Bridges, 1984; Rosenblatt and Ceus, 1998). The absence of estradiol levels during pregnancy impairs the rapid onset of maternal care (Numan and Insel, 2003). In ERαKO and ERKO mice, females display increased time to respond to pups and increased rates of infanticide (Ogawa et al., 1998; Kuroda et al., 2011; Imwalle, Gustafsson, and Rissman, 2005). Furthermore, a single dose of estradiol to virgin females can decrease latency to display maternal care in a sensitization paradigm.

Progesterone starts rising at G3 and levels remain elevated until late pregnancy (G18), when they drastically fall (Garland et al., 1987; Grota and Eik-Nes, 1967; Sanyal, 1978). This increase in progesterone levels is required to maintain pregnancy as well as for the rapid display of maternal care (Bridges, Rosenblatt, and Feder, 1978; Siegel and Rosenblatt, 1978). However, progesterone treatment to virgin female fails to improve mothering (Lonstein et al., 2015) and seems to require the concomitant action of estradiol (Bridges, 1984; Doerr, Siegel, and Rosenblatt, 1981).

Prolactin hormone increases at late pregnancy and is required to allow the mammary gland to start lactation and to stimulate ovarian steroidogenesis (Neville, 2006). During the postpartum period, prolactin levels remain high, decreasing progressively until weaning (Smith, Freeman, and Neill, 1975; Taya and Greenwald, 1982; Taya and Sasamoto, 1981). Prolactin treatment to female rats induces a decrease in maternal sensitization latency (Moltz et al., 1970; Bridges, 1984; Bridges et al., 1985).

Oxytocin is released centrally from early pregnancy to participate in the release of prolactin (Borrow and Cameron, 2012) and to promote the rapid onset of maternal care after delivery (Insel and Harbaugh, 1989; Olazabal and Ferreira, 1997). Among other factors that seem to be important -but not critical- to maternal care are glucocorticoids (Hennessy et al., 1977; Siegel and Rosenblatt, 1978; Brummelte, Pawluski, and Galea, 2006), opioids (Rubin and Bridges, 1984) and vasopresin (Bosch and Neumann, 2012).

5.3 The maternal brain network

The fact that hormonal changes during pregnancy and that continuous exposure to pups induces maternal care suggests that there may be a brain network that, while being activated by hormonal and sensorial stimulus, ultimately triggers mothering. An ”approach / avoidance” model of mothering has been proposed (Rosenblatt and Mayer, 1995; Lonstein et al., 2015) (Fig.5.2. In this model, a default inhibitory network is activated in virgin females triggering pup avoidance. At pregnancy, hormonal changes activate an excitatory network, inhibiting avoidance and promoting approach toward pups.

The primary excitatory region of the maternal brain network is the mPoA and the ventral bed nucleus of the stria terminalis (BNSTv)(Lonstein et al., 2015; Kristal, 2009). This region promotes behaviors, integrating environmental cues, activating a mesolimbic dopaminergic pathway and inhibiting the avoidance network (Fang et al., 2018). The inhibitory regions involved in avoidance toward pups are the median amygdala (MeA) and their connections to the ventromedial hypothalamus (VMH) and the anterior hypothalamus (Lonstein et al., 2015; Kristal, 2009).

5.3.1 The mPoA / BSTv

It is well established that the mPoA and its adjacent region BSTv are required for maternal care (Lonstein et al., 2015; Kristal, 2009). mPoA lesion or removal of their afferences before delivery dramatically disrupts several components of mothering, independently of hormonal levels (Numan and Callahan, 1980; Numan, McSparren, and Numan, 1990; Terkel, Bridges, and Sawyer, 1979). In addition, electrical stimulation of the mPoA in nulliparous females decreases latency in the maternal sensitization test (Morgan et al., 1999b). Maternal sensitization also produces a long-lasting activation of *cfos* expression in the mPoA (Kalinichev et al., 2000; Lonstein et al., 1998; Numan and Numan, 1994; Fleming and Walsh, 1994).The components of mothering affected by mPoA lesions includes licking, grooming, nest building and retrieving pups but not nursing (Lonstein et al., 2015). Only a small subset of efferences connecting the mPoA to the periaqueductal gray area (PAG) are known to be involved in nursing behaviors (Walker et al., 2004; Lonstein and Stern, 1997). Among the mPoA, morphological changes happen during pregnancy that activate the maternal network, we can find the increase in glial cell numbers (Featherstone, Fleming, and Ivy, 2000) and neural dendritic branches (Keyser-Marcus et al., 2001). These changes introduce permanent organizational modifications in the mPoA that facilitate mothering with ulterior litters (Keyser-Marcus et al., 2001).

The mPoA region, through the action of the high density of hormonal receptors such as estrogen, prolactin, progesterone and oxytocin, integrates the endocrine state of the animal to promote maternal care postnatally. For instance, stereotaxic inactivation of ERα with a siRNA in the mPoA drastically impairs maternal care (Ribeiro et al., 2012). In a recent study, optogenetic inactivation of mPoA *Esr1*-positive cells impaired pup approach and retrieval, whereas activation of this cell population promoted maternal care (Fang et al., 2018). Interestingly, recording of mPoA neurons during the episodic sequences of maternal care identified, activity peaks around at the beginning of each active phase, prior to pup retrieval, suggesting that mPoA neurons may trigger this behavior (Fang et al., 2018). Concerning prolactin, direct prolactin infusion into the mPoA in nulliparous females accelerates maternal sensitization (Bridges et al., 1990; Bridges et al., 1997). The action of oxytocin may be indirect as no oxytocin is released in the

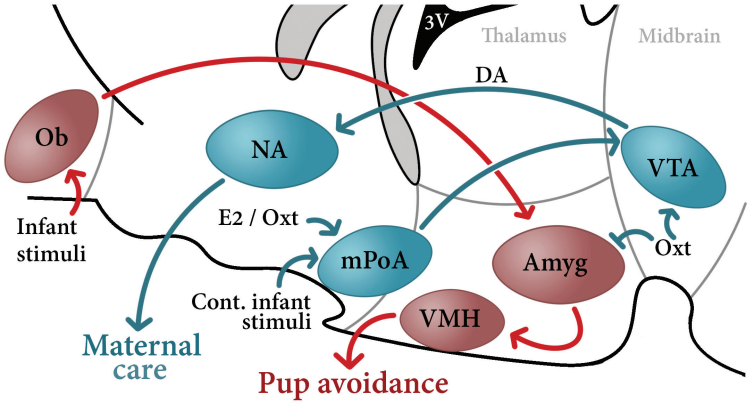


Figure 5.2. Model of the maternal brain network in the female rodent. Natural avoidance toward pups found in virgin females occurs through the activation of the amygdala. Maternal sensitization (continuous interactions with pups) or pregnancy activates the mPoA. The mPoA activates in turn a midbrain pathway of dopaminergic neurons promoting maternal care. This network involves the release of dopamine by the ventral tegmental area (VTA) to the nucleus accumbens (NA). ob= olfactory bulbe, vmh= ventromedial hypothalamus.

mPoA through lactation (Bosch et al., 2010). However, oxytocin receptor expression in the mPoA is associated with licking and grooming (Francis, Champagne, and Meaney, 2000).

The mPoA receives sensorial information that is integrated jointly with other non-hormonal factors to promote a maternal response. The activity of the mPoA changes concomitantly with the changing needs of the offspring during the neonatal period. While at birth the mPoA is fully activated and plays a facilitatory role in maternal care, during late lactation the mPoA activity decreases promoting the independence of pups (Pereira and Morrell, 2009; Pereira and Morrell, 2011). This effect can be reversed when a new litter is introduced (Uriarte et al., 2008) suggesting the importance of sensorial stimulation of pups to trigger maternal care postnatally.

5.3.2 The mPoA-VTA-NA maternal motivation network

The mPoA has efferences connecting to the mesolimbic dopaminergic (DA) system, network involved in maternal motivation (Numan and Stolzenberg, 2009). The mesolimbic DA system includes the ventral tegmental area (VTA) and pro-

jections, among other regions, to the nucleus accumbens (NA). The NA is known to be involved in reward and motivation (Berridge, 2004; Voorn et al., 2004). Studies have suggested a role of each individual region in maternal behavior. Specifically, DA released into the NA by microdyalisis enhances maternal care (Hansen, Bergvall, and Nyiredi, 1993) and pup presentation to a female after delivery increases DA release into the mPoA (Champagne et al., 2004). After delivery, mothers displays increased levels of dopamine, serotonin and vasopressin in the mPoA compared to nulliparous females (Olazábal et al., 2004; Lonstein et al., 2003). Pharmacological inhibition of the dopamine receptors D1 and D2 in this region disrupts different components of maternal care (Vernotica, Rosenblatt, and Morrell, 1999; Miller and Lonstein, 2005; Numan et al., 2005; Stolzenberg et al., 2007). The VTA is activated by maternal care and VTA lesions impair several components of mothering, presumably by disabling the release of DA to the NA (Hansen, 1994; Numan and Smith, 1984; Stack et al., 2002). A recent study has demonstrated the importance of this dopaminergic pathway. mPoA *Esr1*-positive cells that project to the VTA, indirectly stimulate DA release by inhibiting GABAergic neurons (Fang et al., 2018), known to inhibit DA release (Tan et al., 2012).

In this motivation network, dopamine release is not only related to pup retrieval but also to differences in licking and grooming. High licking and grooming mothers (HLG) have a greater release of DA and greater number of DA receptors in the NA compared to low licking and grooming (LLG) mothers (Champagne et al., 2004).

5.3.3 Non-genomic transmission of maternal care: multigenerational inheritance

A series of seminal studies in the field demonstrated that females that experienced high or low licking and grooming repeat the same phenotype toward their own offspring's (Champagne et al., 2001; Champagne et al., 2003). The transmission of these naturally-occurring variations in maternal behavior occurs through organizational changes in the mPoA early during development. For instance, pups experiencing LLG have a reduction of ERα as well as oxytocin receptors in the mPoA that lasts across their lifespan (Peña, Neugut, and Champagne, 2013).

Moreover, when these females were injected with EB, no cfos expression was detected in the mPoA, suggesting that LLG offsprings are less responsive to estrogen action than HLG offsprings. Additionally, LLG reduces the mRNA expression of the DA receptors D1, D2 and D3 in the NA of the offspring (Peña et al., 2014). By using a cross-fostering paradigm, it was demonstrated that the experience of being with a LLG or a HLG dam during the first 6 days of postnatal life is critical, as the effects could not be reversed after that time (Peña, Neugut, and Champagne, 2013). Reduction of ERα expression in LLG pups was found to involve changes at the chromatin level. For instance, pups raised by a LLG mother have decreased recruitment of Stat5b binding at the *Esr1* gene promoter, which is associated with H3K9me3, a repressive histone mark (Champagne and Meaney, 2006). Moreover, *Esr1* gene promoter was found to be hypermethylated in LLG pups compared to pups raised by a HLG dam (Peña, Neugut, and Champagne, 2013).

These findings demonstrate that differences in maternal care shape the brain during development. The consequences of these organizational changes in the brain are not restricted to maternal care transmission. Differences in licking and grooming have an impact in offspring reproduction (Cameron et al., 2008), cognition (Liu et al., 2000), stress responsiveness (Meaney and Szyf, 2005), social interactions (Parent and Meaney, 2008) and sexual behavior (Cameron, Fish, and Meaney, 2008; Sakhai, Kriegsfeld, and Francis, 2011), among others. Only one study has addressed the question on the link between maternal care and sexual maturation. Cameron et al. (2008) demonstrated that pups raised by LLG dams experienced early puberty compared to those raised by HLG mothers. Concerning the reproductive parameters, authors observed effects opposed to what we would expect. Female pups raised by HLG dams were less sexually receptive and they were less responsive to estrogen-induced LH surge (Cameron et al., 2008). Moreover, HLG-raised pups also release fewer oocytes and were less likely to become pregnant (Cameron, Fish, and Meaney, 2008; Sakhai, Kriegsfeld, and Francis, 2011; Uriarte et al., 2007)

As the differences in maternal care are transmitted across generations, we would expect to find the related individual consequences in behaviors to persist multigenerationally. Evidence supporting this hypothesis is provided from studies of stress responsiveness. Glucocorticoid activity and hypothalamic pituitary

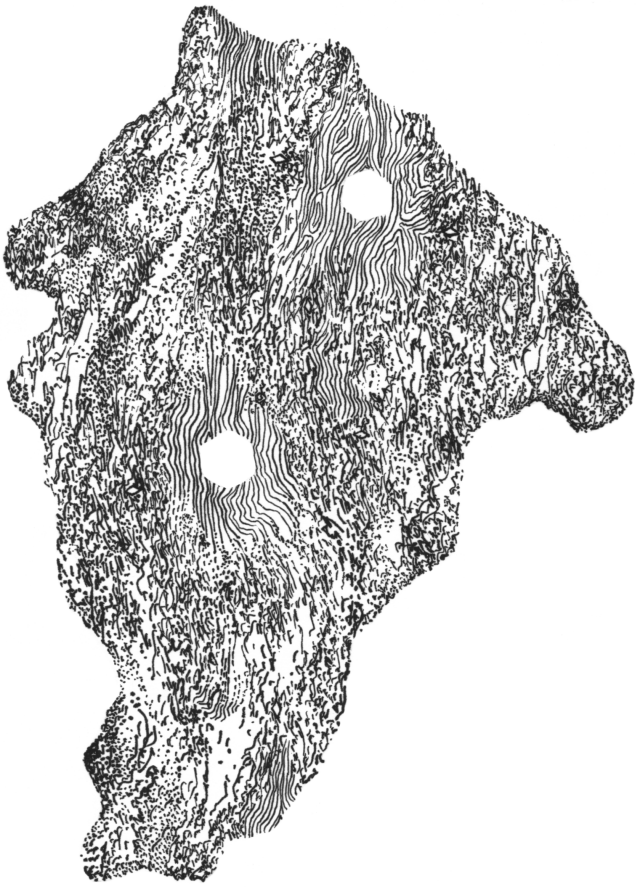
adrenal axis (HPA) response to stress are increased in pups raised by LLG dams (Meaney and Szyf, 2005). This effect was found to be induced by an hypermethylation at the *Nr3c1* promoter in the hippocampus, gene encoding for the glucocorticoid receptor. Interestingly, changes in stress responsiveness persisted from the one generation to the next (Francis et al., 1999). Moreover, the idea of being the germ cell the causal factor of this transmission was discarded by a cross-fostering paradigm. Specifically, the biological offsprings of LLG dams reared by HLG dams were found to be less fearful than offsprings reared by LLG dams (Francis et al., 1999). Overall, these findings suggest that maternal care shapes the brain and alters behavior via nongenomic transmission. Concerning sexual maturation, as we would expect to find variations in pubertal onset caused by LLG/HLG to persist across generations, no studies have addressed this question so far.

5.4 Endocrine disruption of maternal behavior

Few studies have addressed the effects of EDCs on maternal care. A gestational or adult exposure to BPA to 10µg/kg/d in CD-1 mice lead to a decrease in nursing and increased out-nest activities (Palanza et al., 2002). In the same line, gestational exposure to the estrogenic compound MXC decreases the time dams spent nursing pups during the dark cycle and increases the time outside the nest (Palanza et al., 2002). Time in out-of-nest activities is widely associated with reduced maternal motivation (König and Markl, 1987). Interestingly, Palanza et al. (2020) showed that the combination of gestational and adult exposure did not produce any adverse effect. The alteration in nursing found in these studies suggests a decrease in the neuroendocrine system related to prolactin release.

Only one study has found significant alterations in licking and grooming behavior. For instance, adult BPA exposure to 0.04 mg/kg/d starting from mating decreases the time dams spent liking the offspring (Seta et al., 2005). Such decrease was similar for male and female pups. Reduced maternal licking in male pups is associated with reduced sexual and social behaviors during adulthood (Moore, 1984). While this study did not use a mechanistical approach, reduced licking and grooming could be due to alterations in estrogen and oxytocin signaling in the preoptic area or to a decrease in the dopaminergic system related to maternal motivation.

SCOPE OF THE THESIS



6 | Scope of the thesis

In our introduction we aimed at summarizing current knowledge regarding the hypothalamic control of female puberty, reproduction and maternal behavior in the context of endocrine disruption. Although a substantial number of studies indicates an effect of EDCs in reproduction and behavior, but the main molecular and cellular mechanisms remains unknown, especially in the context of transgenerational inheritance. The aim of this thesis is to determine the effects and mechanisms of adult or developmental exposure to EDCs on the hypothalamic control of reproduction and maternal behavior.

In the **first experimental study**, we tested the effects of two doses of BPA on the hypothalamic control of reproduction in female rats. We tested a low environmental dose of 25ng/kg/d and a high contrasting dose of 5mg/k/d during 15 days using daily s.c injections. We compared 2 windows of exposure: early postnatal (PND 1 to 15) or adult exposure. We studied estrous cyclicity,and folliculogenesis and characterized pulsatile GnRH secretion and the LH preovulatory surge in order to identify potential neuroendocrine mechanisms of disruption. Neonatal exposure to these two doses of BPA had been previously shown to affect pubertal timing and GnRH neuron maturation by altering GABAergic signaling before puberty (Franssen et al., 2016). In adult females, folliculogenesis, GnRH and LH preovulatory surge measurements were carried out 24h and 30 days after exposure. Additionally, transcriptional expression of gene related to reproduction and circadian rhythm was measured in hypothalamic explants. In this study, we aimed at answering the following questions:

1. Does exposure to a low dose of BPA during the neonatal period alter the

organization of estrous cyclicity in adulthood?

2. Does BPA exposure during adulthood, outside of the critical organization window of exposure, alter estrous cycle and follicle development?

    - Is the reproductive phenotype reversible?
    - What are the central neuroendocrine mechanisms explaining these effects?
    - Do the two doses of BPA differentially affect the reproductive function?

In the **second experimental study**, we tested the effect of a complex EDC mixture on female sexual maturation and estrus cycle and maternal behavior across generations. EDC mixture was administered orally and daily from two weeks before gestation to the end of lactation. Sexual maturation and function were evaluated by studying pubertal timing, estrous cyclicity, folliculogenesis and GnRH secretion. Maternal care was evaluated through daily recording of in-nest and off-nest spontaneous behavior. Maternal care and reproductive functions were characterized down to the fourth generation. Transcriptomic (RNAseq and qPCR) and epigenetic (ChIP and bisulfite sequencing) analysis were carried out in the mediobasal hypothalamus of F1 and F3 females at P21 in order to identify potential molecular mechanisms. We aimed at answering the following questions:

1. Does an exposure to a mixture of EDCs disrupt sexual maturation and estrous cyclicity across generations?

    - What are the epigenetic/transcriptional mechanisms explaining potential alterations of the hypothalamic control of puberty and reproduction?

2. Does exposure to an EDC mixture disrupt maternal care across generations?

    - Is this behavioral phenotype transmitted via multigenerational mechanisms?

- Does the potential alteration in maternal care involve alterations of the brain regions involved in maternal care?

3. Could the maternal and reproductive phenotypes be related?

EXPERIMENTAL STUDY

Persistent vs Transient Alteration of Folliculogenesis and Estrous Cycle After Neonatal vs Adult Exposure to Bisphenol A

7 Experimental study One

Persistent vs Transient Alteration of Folliculogenesis and Estrous Cycle After Neonatal vs Adult Exposure to Bisphenol A

Contents

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Abstract

Exposure to bisphenol A (BPA), a ubiquitous endocrine-disrupting chemical (EDC), is known to produce variable effects on female puberty and ovulation. This variability of effects is possibly due to differences in dose and period of exposure. Little is known about the effects of adult exposure to environmentally relevant doses of this EDC and the differences in effect after neonatal exposure. This study sought to compare the effects of neonatal vs adult exposure to a very low dose or a high dose of BPA for 2 weeks on ovulation and folliculogenesis and to explore the hypothalamic mechanisms involved in such disruption by BPA. One-day-old and 90-day-old female rats received daily subcutaneous injections of corn oil (vehicle) or BPA (25 ng/kg/d or 5 mg/kg/d) for 15 days. Neonatal exposure to both BPA doses significantly disrupted the estrous cycle and induced a decrease in primordial follicles. Effects on estrous cyclicity and folliculogenesis persisted into adulthood, consistent with a disruption of organizational mechanisms. During adult exposure, both doses caused a reversible decrease in antral follicles and corpora lutea. A reversible disruption of the estrous cycle associated with a delay and a decrease in the amplitude of the LH surge was also observed. Alterations of the hypothalamic expression of the clock gene *period circadian protein homolog 1 (Per1)* and the reproductive peptide phoenixin indicated a disruption of the hypothalamic control of the preovulatory LH surge by BPA.

7.1 Introduction

Bisphenol A (bisphenol A (BPA)) is a ubiquitous EDC used in the production of polycarbonate plastics and epoxy resins (Vandenberg, Hauser, Marcus, Olea, and Welshons, 2007). Despite its partial ban in some countries, it is currently one of the most largely used chemical compounds in the world with more than 8 billion tons produced each year (Greiner, Kaelin, and Toki, 2004). Human exposure is nearly universal in developed countries and occurs mainly through contaminated beverages and food (Kang, Kondo, and Katayama, 2006). Several studies indicate widespread contamination of fetuses and neonates, leading to the questions as to whether such an EDC can affect development (Ikezuki et al., 2002; Nachman et al., 2015; Schonfelder et al., 2002) and whether there is a limit for a safe exposure. Currently, the EPA "safety level" of BPA is set at 50 μg/kg/d i.e. 1,000 times the average human exposure (Vandenberg et al., 2007). The European Food Safety Authority's tolerable daily intake was recently lowered to 4 μg/kg./d.(EFSA, 2015).

Sex steroids play a crucial role perinatally in "organizing" the control of female reproduction. For that reason, the adult female estrus cycle is altered following exogenous exposure to sex steroids during that vulnerable perinatal period (Nozawa et al., 2014). Therefore, the effects of early exposure to BPA on puberty and reproduction are a matter of concern. Recent evidence suggests that exposure to BPA during this sensitive developmental period could have long-term impacts on reproductive function (Gore et al., 2015). Early exposure to BPA affects puberty onset with effects depending markedly on the window and dose of exposure, and possible non-linear dose–response relationship (Adewale et al., 2009; Fernandez et al., 2009; Franssen et al., 2016; Honma et al., 2002; Howdeshell et al., 1999; Laws et al., 2000; Losa-Ward et al., 2012; Murray et al., 2007; Nagao et al., 1999; Nah, Park, and Gye, 2011; Nikaido et al., 2004; Nikaido et al., 2005b; Parent et al., 2015; Parent et al., 2016; Tinwell et al., 2002; Yu et al., 2011). Effects of neonatal exposure to BPA on estrous cyclicity have produced inconsistent results. While a few studies did not show any effect on estrous cyclicity (Moore-Ambriz et al., 2015; Santamaria et al., 2016; Vigezzi et al., 2015; Xi et al., 2011), others reported abnormal cyclicity (Adewale et al., 2009; Delclos et al., 2014; Fernandez

et al., 2009; Lee et al., 2013; Mendoza-Rodriguez et al., 2011; Monje et al., 2010; Nah, Park, and Gye, 2011). Prenatal or perinatal exposure to BPA also decreases the number of preantral follicles in mice (Mahalingam et al., 2017) and rats (Patel et al., 2017) and leads to a decline in fertility and fecundity(Cabaton et al., 2011). Taken together, these studies suggest that prenatal or neonatal exposure to BPA doses in the mg or μg ranges affects several structures and functions of the neuroendocrine system and the ovaries. Whether the exposure to very low doses of BPA neonatally or during adulthood results in different effects on female neuroendocrine and ovarian functions remains largely unknown.

We have recently shown that neonatal exposure to a very low dose of EPA (25 ng/kg/day) delays the developmental changes in GnRH secretion before puberty while a high dose of EPA (5 mg/kg/day) results in early occurrence of those neuroendocrine changes (Franssen et al., 2016). This effect is followed by a delayed or advanced vaginal opening after exposure to the low or high dose of EPA, respectively. Here, the aim is to elucidate whether such a neonatal exposure to a very low dose of EPA could produce persistent disruption of folliculogenesis and estrous cycle that could be consistent with disturbed organization. We also used the high EPA dose since opposing effects on acGnRH secretion and pubertal timing were seen after using the low and the high doses of EPA neonatally (Franssen et al., 2016). Finally, the aim is to evaluate whether adult EPA exposure in similar conditions would produce persistent or transient effects on ovulation and folliculogenesis.

7.2 Methods

Animal care and exposure

Adult Female Wistar rats from the animal facility of the University of Liège were housed individually in standardized conditions (12h dark/light phase from 4pm, 22.8°C and food and water *ad libitum*). All animals were raised in BPA-free cages (Polypropylene cages, Ref 1291H006, Tecnilab, Netherlands) and fed EDC and phytoestrogen free chow (V135 R/Z low phytoestrogen pellets, SSNIFF Diet, Netherlands). Water was supplied in glass bottles.

Two timings of exposure were studied: female rats were exposed neonatally or during adulthood. Neonatal exposure to BPA started on PND 1 and ended on PND 15. Animals received a daily s.c injection (0.05 ml) of corn oil (vehicle) or one of the 2 doses of BPA, a low environmental dose of BPA dose of 25 ng/kg/d or a high dose of 5 mg/kg/d (BPA Ref: 23,9658; Sigma–Aldrich, Saint Louis, USA).BPA was diluted in ethanol at an initial concentration of 100 mg/ml and then diluted in corn oil. The same concentration of ethanol was added to the control solution of corn oil. Once we obtained the final solution, tubes were opened to allow ethanol evaporation. Subcutaneous injections were given every 24h between 10.00 and 12.00 am. Adult exposure took place from PND 90 to PND 105 under the same conditions. All experiments were carried out with the approval of the Belgian Ministry of Agriculture and the Ethical Committee at the University of Liège.

Experimental design

Effects of exposure to acBPA from PND 1 to 15 on estrous cycle and folliculogenesis

Litters were homogenized for size and sex ratio on the first postnatal day of life in order to have 10-12 pups per litter and a 1:1 male:female ratio. Cross-fostering of maximum 2 pups per litter was used when homogenization was required. The day of birth was considered as PND 0. Pups were weaned on PND 21. Twenty-eight female pups born from 8 dams were exposed from PND 1 to 15 to 25 ng/kg/d (n=7) or 5 mg/kg/d of BPA (n=7) or corn oil (vehicle) (n=14). The animals were followed for estrous cyclicity from the time of vaginal opening until PND 105

when they were sacrificed to study ovarian folliculogenesis during the diestrus stage.

Effects of exposure to BPA from PND 90 to PND 105 on estrous cyclicity, GnRH and LH secretion, hypothalamic gene expression and folliculogenesis

Eighty-one female rats were followed for estrous cyclicity from PND 60 onwards. Among these females, only those (n = 74) showing at least 3 regular cycles out of 4 consecutive cycles at PND 90 were selected for the exposure experiment. These adult female rats were exposed to 25 ng/kg/d of BPA (n = 27) or 5 mg/kg/d (n = 26) or corn oil (n = 21) for 15 days from PND 90 to PND 105. A group of females in diestrus were sacrificed 24h after the last dose of BPA or corn oil to measure plasma and pituitary LH and FSH levels (CTL: n = 9; BPA-25 ng: n = 12; BPA-5 mg: n = 7), hypothalamic gene mRNA expression (CTL: n = 5; BPA-25 ng: n = 6; BPA-5 mg: n = 5) as well as folliculogenesis (n = 6/group). Only females in diestrus based on smear results were considered for analysis.

GnRH pulse frequency was analysed *ex vivo* 24h after the last dose of BPA by using a hypothalamic explant incubation (n = 4/group). Only females on diestrus were considered for analysis. Another group of BPA-exposed female rats was followed for estrous cyclicity (BPA-25 ng: n = 11; BPA-5 mg: n = 11) until four weeks after the exposure. Among those females, a subgroup undertook serial blood samples in order to determine LH surge (BPA-25 ng: n = 5; BPA-5 mg: n = 5). Finally, a set of females was sacrificed 4 weeks after exposure and one ovary per animal was collected to study folliculogenesis on diestrus (CTL: n = 6; BPA-25 ng: n = 6; BPA-5 mg: n = 6).

Estrous cyclicity

Animals exposed from PND 1 to PND 15 were examined daily to evaluate estrous cyclicity from the day of vaginal opening until PND 105. Estrous cyclicity was measured with vaginal smears taken every day in the beginning of the afternoon as described previously (Franssen et al., 2014). Rats exposed during adulthood were examined for estrous cyclicity from 2 weeks before the exposure to 4 weeks after the end of exposure to BPA.

We defined a regular cycle as a sequence of diestrus 1, diestrus 2, proestrus and estrus in 4 consecutive days (Goldman, Murr, and Cooper, 2007). The per-

centage of females having a regular cycle and the time spent in every stage of the cycle were calculated every period of 8 days corresponding to 2 full estrous cycles.

Hypothalamic explant incubation and GnRH assay

As previously shown, a neonatal exposure to BPA significantly affects GnRH pulse frequency at PND 20 (Franssen et al., 2016). In order to determine whether GnRH frequency was affected after adult BPA exposure, GnRH secretion from hypothalamic explant was studied *ex vivo* 24h after the last s.c injection of BPA. As previously described (Matagne et al., 2004; Bourguignon and Franchimont, 1984a), this method allow to reliably measure GnRH pulsatility. Briefly, after decapitation, the brain was placed ventral side up. Two sagittal incisions along the lateral hypothalamic sulci and two transversal incisions of 2 mm were made 2 mm ahead from the anterior boundaries of the optic chiasm and along the caudal margin of the mammillary bodies. Then, the hypothalamic region including both the mediobasal hypothalamus (MBH) and the mPoA were transferred into an individual chamber, in a static incubator, submerged in MEM. The incubation medium was collected and renewed every 7.5 min for a period of 4 hours. The GnRH released into the incubation medium was measured in duplicate using a radioimmunoassay method with intra and inter-assay coefficients of variation of 14 and 18% respectively. The highly specific CR11-B81 rabbit anti-GnRH antiserum (1:80,000) was kindly provided by Dr. V.D. Ramirez (Urbana, IL)(Dluzen and Ramirez, 1981). Data below the limit of detection (5 pg/7.5-min fraction) were assigned that value.

Serum and pituitary LH and FSH radioimmunoassay

Blood samples and pituitaries were quickly collected 24h after the end of adult exposure to BPA or corn oil. Blood samples were stored overnight at 4°C, followed by decantation of serum and stored at −20°C until the assay was performed. Pituitary samples were stored in PBS at −20°C before homogenization with ultrasound and centrifugation to obtain the supernatant used for the assays.

Serum and pituitary LH and FSH levels were determined using a double Ab method and a RIA kit (mLH RIA, rFSH RIA), kindly supplied by NIH (Dr. A.F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA). Antibodies used

were NIDDK-anti-rFSH-S-11 and LH antiserum AFP-240580. Rat FSH antigen NIDDK-rFSH-I (AFP-5178B) and rat LH-I-8 (AFP-12066B) were labelled with ^{125}I by the chloramine-T method, and the hormone concentration was calculated using the mouse LH and rat FSH reference preparation (LH: AFP-5306A; FSH: NIDDK-rFSH-RP-2, AFP-4621B) as standard. The intra- and interassay coefficients were 6-9% and 7-10% for LH and FSH respectively. The sensitivity of the assay was 4 pg/100μl for LH and 0.125 ng/100μl for FSH.

LH surge

Females were handled for habituation to tail-blood sampling daily during 2 weeks prior to the experiment. Pre- and post-exposure blood samples were collected 2 and 4 weeks before and after BPA exposure, respectively. During those periods, samples were collected every hour from 13h00 (3 hours prior to the beginning of the dark cycle) to 22h00 during proestrus defined by vaginal smear. Because cyclicity was disrupted during BPA exposure, blood samples were collected from 11h00 to 22h00 during two consecutive days prior to the expected estrus stage during the second week of exposure LH was measured using an ultrasensitive ELISA LH assay (Steyn et al., 2013). Briefly, 96-well high affinity binding plates (Corning), were coated overnight with a bovine monoclonal antibody (in 0.015M Na_2Co_3 and 0.035M $NaCo_3$ coating buffer, *pH* 9.6). Samples and serial dilution of known concentrations of LH were incubated for 2 hours. After incubation, a rabbit polyclonal primary antibody for LH (1:10,000) a polyclonal goat anti-rabbit IgG secondary antibody (1:1,000; DAKO) and 1-Step^{TM} Ultra TMB-ELISA Substrate (ThermoFisher) were added to each well. The sensitivity of this assay was 0.06 ng/ml and intra- and interassay coefficients of variance were 6.3% and 10% respectively.

For each detected pulse, the amplitude was determined by subtracting the highest LH value from the basal value immediately prior to the onset of the pulse. Overall basal levels of LH secretion were determined by combining a minimum of the 5 lowest LH measurements from each mouse.

Ovarian histology

When the ovaries were removed, they were weighted on PND 105, 90 days after the end of neonatal exposure to BPA or corn oil or 24h after the adult exposure

to BPA. In order to determine whether folliculogenesis was affected by BPA, the ovaries were removed on PND 105 after neonatal exposure to BPA and either on PND 105 or PND 135, corresponding to 24h or 30 days after the end of adult exposure to BPA. The ovaries were fixed in 4% paraformaldehyde overnight, dehydrated in 70% EtOH and paraffin-embedded. For histological analysis, 8 μ coronal sections were cut using a microtome. Every other section was deparaffinized, stained with hematoxylin and eosin, covered with a coverslip and examined for quantification of folliculogenesis.

For quantification, images of every other section throughout the whole ovary were acquired using an automated digital microscopy system DotSlide (Olympus, BX51TF, Aartselaar, Belgium). Dotslide images taken at a magnification of 10x which were in a proprietary format were converted into a standard TIFF format and 3-fold decimated, easier to handle. Thereafter, quantification of follicles and corpora lutea was carried out manually with Aperio ImageScope v12.3.2.8013 software (SCR014311, Leica Biosystems) by an experimenter blinded to treatment. Total ovarian volume was automatically calculated using an original program developed using the image analysis toolbox of the MatLab (SCR001622, 2016a, The Mathworks Inc., Natick, MA, USA) software. Folliculogenesis was analysed by quantifying follicles at every stage of folliculogenesis: primordial, primary, secondary, antral and atretic follicles. In addition, cysts and corpora lutea were identified. The follicles were classified according to well-established criteria (Hirshfield and Midgley Jr., 1978). Double-counting of late stage follicles was avoided by digitally marking each follicle throughout the consecutive images. Each follicle was counted once whenever the oocyte was present. For quantification of early stage follicles (primordial and primary follicles), a 2-fold correction factor was added to compensate for the sections that were not analysed. Measurements are expressed as number of follicles or corpora lutea per volume (mm^3).

Real-time PCR

Because neonatal exposure to BPA altered the expression of hypothalamic genes involved in the GABAergic pathway *glutamic acid decarboxylase 2 (Gad2)* and *glutamic acid transporter 2 (Gat2)* (Franssen et al., 2016),we studied the expression of those genes after adult exposure to corn oil or BPA in the MBH and PoA. The clock genes *Per1*, *Per2*, *Bmal1*and *Clock*, as well as the novel reproduction-

Gene		Sequence	Tm	bp	Accession Number
β-$Actin$	F	$5' - CGCGAGTACAACCTTCTTGC - 3'$	59.6	200	NM_031144.3
	R	$5' - ATACCCACCATCACACCCTG - 3'$	59.1		
$GAT2$	F	$5' - TTCATCGGGCTCATTATGCTCA - 3'$	59.9	193	NM_133623.1
	R	$5' - TGATAAGAGGCCACGGCTTG - 3'$	60.1		
$GAD2$	F	$5' - GCACCTGTGACCAAAAACCC - 3'$	59.9	73	NM_012563.1
	R	$5' - AGGTCTGTTGCGTGGAGAAG - 3'$	60.0		
Cas-3	F	$5' - GGAGCTTGGAACGCGAAGAA - 3'$	57.9	169	NM_012922.2
	R	$5' - ACACAAGCCCATTTCAGGGT - 3'$	57.2		

Table 7.1. Primer Sequence.Tm: Annealing temperature, *bp*: lenght.

related peptide *Pnx*, *Esr1*, *Esr2* and *Kiss1* mRNA levels were also measured in both MBH and PoA. Quantitative PCR (qPCR) analysis was carried out in the MBH and the PoA. After decapitation, the PoA and the MBH were rapidly dissected. The brain was placed ventral side up. The dissection began by 2 sagittal incisions along the lateral hypothalamic sulci. Two transversal incisions of 2 mm were made 2 mm ahead from the anterior boundaries of the optic chiasm and along the caudal margin of the mammillary bodies. Finally, a frontal incision was made 2 mm under the ventral surface of the hypothalamus.

Total RNA was extracted from the MBH, medial PoA and total ovarian tissue using the Universal RNA Mini kit (Quiagen, Netherlands). Prior to extraction, ovarian tissue was homogenized using a Mikro-Dismembrator S (SartoriusStedim, Germany). Five hundred ng of RNA for each sample were reverse transcribed using the Transcriptor first strand cDNA synthesis kit (Roche, Germany). For real-time quantitative PCR reactions, the cDNA of our samples were diluted 10 fold and 4μl were added to a mix of 5μl FastStart Universal SYBR Green Master (Roche, Germany), 0.4μl of nuclease-free water and 0.3μl of forward and reverse primer (see primer sequences in Table 7.1). The samples were run in triplicate using a LightCycler 480 thermocycler (Roche, Germany). *Ct* values were obtained from each individual amplification curve and the average *Ct* was calculated for each target gene in each sample. Quantification of relative gene expression was performed using an original program developed on Python 2.7.13 according to the $\Delta\Delta Ct$ method implemented with the Pfaffl equation which takes into account reaction efficiency depending on primers (Pfaffl, 2001). All assays had efficiencies between 1.9 and 2.1. β-actin was used as housekeeping gene.

Statistics

Data analysed with non-parametric test were expressed in median and interquartile (IQR). Numeric values of data analysed with a two-way ANOVA were expressed as mean ± SEM. When normality and homogeneity of variance were not accomplished, a two-group comparison Mann-Whitney non-parametric test was carried out. When making multiple comparisons, alpha was adjusted by using Bonferroni correction. Effect sizes were calculated using the equation $r = \frac{Z}{\sqrt{N}}$), where the Z is consistent with the adjusted normally distributed variable value. Estrous cyclicity after adult BPA exposure was analysed using a McNemar test comparing the pre-exposure period versus the exposure period (PRE vs EXP) and the exposure period versus the post-exposure period (EXP vs POST) using each group as their own control. When the conditions for this test were not fulfilled, a mid-p McNemar test based on the binomial test was carried out. Estrous cyclicity after neonatal exposure and LH surge data after adult exposure were analysed by using a repeated-measures ANOVA followed by the Tukey's test for multiple comparisons and η^2 and Cohen's d as an indicator of effect size. The level of statistical significance was a *p value* lower than 0.05. Data were analysed using Prism 6.01 (SCR002798, Graph Pad, Inc.).

7.3 Results

Persistent or transient estrous cycle disruption after neonatal or adult BPA exposure

As we had previously shown (Franssen et al., 2016), pubertal onset was affected by neonatal exposure to BPA with opposing effects depending on the dose. Vaginal opening occurred 3 days later on average after neonatal exposure to BPA 25 ng/kg/d and 3 days earlier after BPA 5 mg/kg/d. The time period from the day of vaginal opening (VO) to the first full regular cycle was significantly increased (5.5 and 6.1 days, respectively) after exposure to both the low BPA dose (U = 7, z = 2.0; $p < 0.05$) and the high BPA dose (U = 6.5, z = 2.2; $p < 0.05$; r = 0.8) (Fig.7.1.a left). Subsequently, neonatal exposure to both the low and high dose of BPA significantly decreased the percentage of cycling females (Fig.7.1.a right, see supplemental table in Appendix D.1). At PND 90-105, while the control group showed regular cycles in 71% of the females, the BPA exposed groups showed only 43% (BPA-25 ng) and 14% (BPA-5 mg) of regularly cycling females. While the BPA treated groups showed a trend towards less time spent in proestrus and more time in diestrus, most values were not significantly different except on PND 82-89 when comparing CTL and the high BPA dose (Fig.7.1.c-e; see Supplemental table in appendix D.1). We aimed at comparing the effects of neonatal exposure to BPA on estrus cycles obtained above with effects observed during and after adult exposure. Adult exposure to BPA for 15 days caused significant alterations of the estrous cycle (Fig.7.1.b and Table 7.2). During exposure to 25 ng/kg/d or 5 mg/kg/d of BPA, the proportion of females with regular cycle decreased markedly and similarly to 12% and 9%, respectively (X^2 = 13.1 and 12.1, p<0.001). This effect persisted for one week after the end of BPA exposure. Subsequently, the percentage of regularly cycling females was restored to 100% four weeks after the end of exposure to the two doses of BPA, indicating the reversibility of the effect. The alteration in cyclicity was characterized by a significant decrease of the time spent in proestrus (BPA-25 ng: X^2 = 12.5, p <0.001; BPA-5 mg: X^2 = 16.1, p <0.001) as well as an increase of the time spent in diestrus (BPA-25 ng: X^2 = 8.5, p <0.01; BPA-5 mg: X^2 = 4.92, p <0.05) (Fig.7.1.c-e). Time spent in estrus was not affected by exposure to BPA. Time spent in proestrus was restored

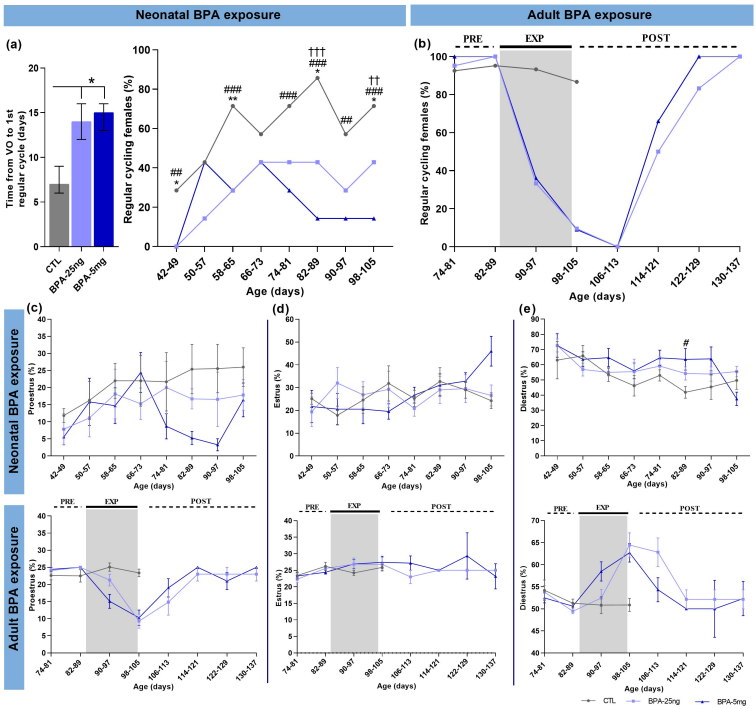


Figure 7.1. Characteristics of estrous cycle after neonatal (PND 1 to 15) and adult (PND 90 to 105) exposure to BPA (25 ng/kg/d or 5 mg/kg/d) or corn oil (CTL). (a): Left: Average time from day of vaginal opening to first regular cycle. Right: Percentage of females exhibiting regular cycling from 42 to 105 days of age after neonatal BPA exposure (n=7/group). (b): Percentage of females exhibiting regular cycling 2 weeks before, during (CTL: n=15; BPA-25ng: n=22; BPA-5mg: n=21) and 4 weeks after adult BPA exposure (BPA-25ng: n=11; BPA-5mg: n=11). (c-e): Percentage of time spent in proestrus (c), estrus (d) and diestrus (e) after neonatal (top) or adult (bottom) exposure to BPA. Neonatal BPA exposure data was analysed using a repeated-measures ANOVA (Tukey's test for multiple comparisons); adult exposure comparisons were analysed using the mid-p McNemar test. Data are expressed as mean [IQR] (a) and percentage $\pm$ SEM (c-e). CTL vs BPA-25ng: $*p < 0.05$, $**p < 0.01$; CTL vs BPA-5mg: $\#\#p < 0.01$, $\#\#\#p < 0.001$. BPA-25ng vs BPA-5mg: $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger$ p<0.001.

		PRE vs BPA						BPA vs POST					
	Group	*n*	=	<	>	z	*p*	*n*	=	<	>	z	*p*
Regular cycling	Corn oil	15	8	3	4	0.57	-	-	-	-	-	-	-
females	BPA-25ng	22	7	15	0	13.06	***	6	1	5	0	7.2	**
	BPA-5mg	21	7	14	0	12.07	***	6	1	5	0	7.2	**
Proestrus	Corn oil	15	8	2	5	2.29	-	-	-	-	-	-	-
	BPA-25ng	22	4	17	1	12.50	***	6	2	4	0	6.25	-
	BPA-5mg	21	3	18	0	16.06	***	6	3	3	0	5.33	-
Estrus	Corn oil	15	12	1	2	1.33	-	-	-	-	-	-	-
	BPA-25ng	22	17	1	4	3.20	-	6	6	0	0	-	-
	BPA-5mg	21	16	2	3	0.80	-	6	4	2	0	0.5	-
Diestrus	Corn oil	15	9	2	4	1.50	-	-	-	-	-	-	-
	BPA-25ng	22	5	15	2	8.47	**	6	3	3	0	5.33	-
	BPA-5mg	21	8	11	2	4.92	*	6	2	4	0	2.25	-

Table 7.2. The number of regular cycles and the number of days spent in proestrus, estrus and diestrus were quantified in each three periods: pre-exposure (PRE), exposure (BPA) and post-exposure (POST). A McNemar test was carried out for comparing treatment versus pre-treatment and a mid-p McNemar test based on the binomial test was used for comparing treatment versus post-treatment. "=" number of females that did not show differences between any of both compared periods. "<" number of females that have shown a decrease in regular cyclicity. ">" number of females that have shown an increase in regular cyclicity. z = McNemar or mid-p McNemar statistical value. p = p-value. n = sample size. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are expressed as number of animals in each group.

to 23-25% after the end of exposure to both doses but EXP vs POST comparisons by using the mid-p McNemar test did not reach significance. Ninety-two percent of the control females showed regular cycles during the pre-exposure (PRE) and the exposure periods (EXP).

Neonatal or adult BPA exposure alters early or late stages of folliculogenesis, respectively

The number of primordial follicles in the ovaries evaluated at PND 105 in the control group was significantly decreased after neonatal exposure to both the low dose (U = 6.0, z = 3.4, p <0.001, r = 1.5) and the high dose (U = 9.0, z = 2 .7, p <0.001, r = 1.2) (Fig.7.2.a). Moreover, the number of atretic follicles per ovary was increased after neonatal exposure to the high dose of BPA (U = 12.0, z = -2.4, p <0.05, r = 1.1). The low dose of BPA did not affect the number of atretic folli-

cles. Cystic follicles were absent in the control ovaries and present after neonatal exposure to both doses of BPA. Ovarian weight was significantly reduced after neonatal exposure to the low BPA dose ($U = 6.5$, $z = 2.2$, $p < 0.05$, $r = 0.8$). While a similar average weight reduction was observed after exposure to the high BPA dose, difference was not found to be significant (Fig.7.2.b).

The number of antral follicles was significantly decreased 24 hours after the last day of adult exposure to the low dose ($U = 0.0$, $z = 2.5$, $p < 0.05$, $r = 1.1$) or high dose of BPA ($U = 0.0$, $z = 2.3$, $p < 0.05$, $r = 1.0$) (Fig.7.2.c). The number of corpora lutea was also decreased after exposure to BPA, however only significantly for the high BPA dose ($U = 0.0$, $z = 2.5$, $p < 0.05$, $r = 1.1$). The number of atretic follicles tended to increase though not significantly in the animals exposed to the two doses of BPA compared to controls. Cystic follicles were found in the ovaries of females exposed to the low and the high doses of BPA but were never observed in control animals (Fig.7.2.d). The effect of BPA on ovarian histology was reversible, since, 30 days after BPA exposure, the number of follicles per volume at the different stages of folliculogenesis was no longer significantly different among the control and exposed animals (Fig.7.2.e). However, the animals previously exposed to the low and high BPA dose still showed cystic follicles which were not observed in the control group (Fig.7.2.e).

Adult BPA exposure transiently disrupts LH surge

Basal serum and pituitary levels of LH and FSH measured during diestrus, 24h after the last day of adult exposure (Fig.7.3.a-d) were not significantly affected. Because the proestrus timing appeared to be disrupted by BPA exposure, we characterized the spontaneous LH surge before, during and after a 15-day exposure to BPA. LH secretion was measured during 2 consecutive afternoons 48 hours after estrus in order to identify the spontaneous LH surge. The LH surge was significantly delayed during the exposure to both doses of BPA (see representative profile in (Fig.7.3). A systematic delay of 2.6 and 2.8 hours on average was observed during exposure to BPA-25ng ($p<0.05$) and BPA-5mg group ($p<0.01$) respectively (Fig.7.3.f). The timing of the LH surge was restored one month after the end of exposure. Additionally, the high BPA dose significantly blunted the LH surge during the second week of exposure compared to the pre-exposure period ($p<0.01$) (Fig.7.3.g). The effect was reversible as the LH surge amplitude

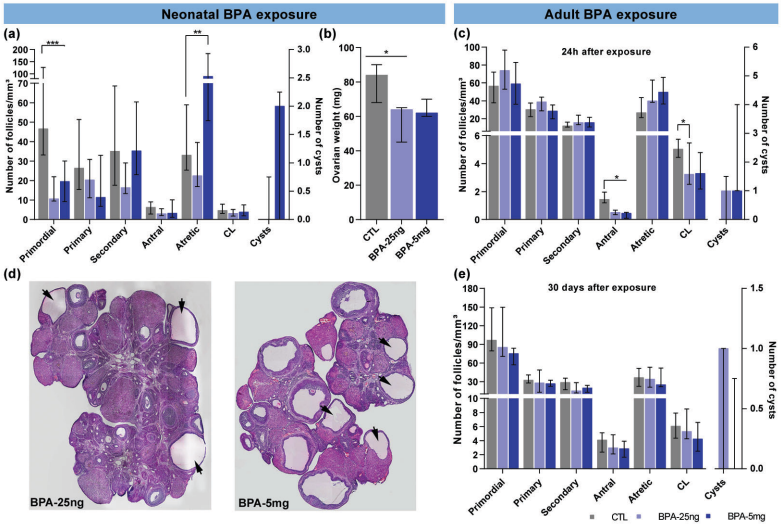


Figure 7.2. Effects of neonatal (PND 1 to 15) and adult (PND 90 to 105) exposure to BPA (25 ng/kg/d or 5 mg/kg/d) or corn oil (CTL) on ovarian weight and folliculogenesis during diestrus. Quantification of follicles and corpora lutea (a) and ovarian weight (b) after neonatal BPA exposure (n=7/group). Quantification of follicles and corpora lutea 24 hours (c) or 30 days (e) after adult BPA exposure (n=6/group). Representative ovarian sections obtained from animals 24h after adult exposure (d). Arrows depict the presence of some cystic follicles. Follicles were quantified in every other section and normalized by ovarian volume (mm3). * p<0.05, ** p<0.01, *** p<0.001 vs CTL. The data was analysed using Mann-Whitney test and represented as median and IQR.

was restored after exposure. The amplitude of the LH surge was not significantly affected by the low BPA dose. We have shown previously that neonatal exposure to BPA disrupts GnRH pulsatile secretion (Franssen et al., 2016). In the current study, we studied the effects of adult exposure to BPA on GnRH pulsatile secretion. Hypothalamic explants obtained from females in diestrus 24h after the end of exposure to BPA were incubated individually in order to study pulsatile GnRH secretion (Fig.7.3.h). GnRH interpulse interval was not significantly different between the control group (41.1±0.2 min) and BPA exposed females to the contrary of what was observed after neonatal exposure (Franssen et al., 2016). GnRH interpulse interval was however significantly longer after exposure to the low dose when compared to the high dose of BPA ($U = 0.0$, $z = 2.2$, $p < 0.05$, $r = 0.5$), with an average difference of 3.1 minutes.

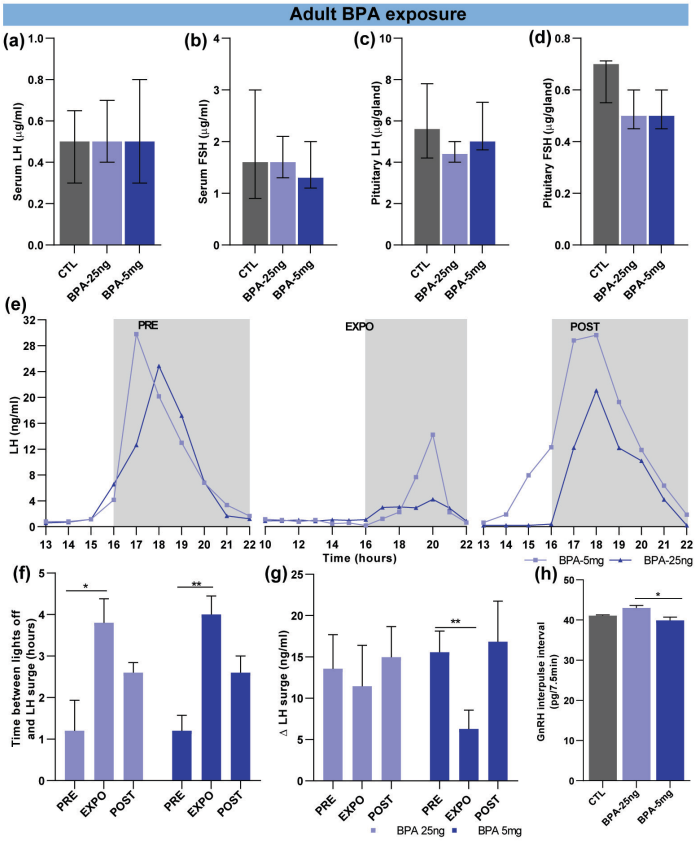


Figure 7.3. Effects of adult (PND 90 to 105) exposure to BPA (25 ng/kg/d or 5 mg/kg/d) or corn oil (CTL) on serum and pituitary LH and FSH, GnRH pulsatile secretion and the preovulatory LH surge. Serum (a-b) and pituitary (c-d) LH (diluted 2000x) and FSH (diluted 500x) levels. Pituitary LH and FSH were multiplied by their dilution factor to obtain an amount of μg/gland (CTL: n=9; BPA-25ng: n=12; BPA-5mg: n=7). Samples were collected 24h after the last BPA or corn oil administration during diestrus stage. (e) Representative LH surge from 2 females exposed to either the low or high BPA dose. The grey area represents the dark phase. (f) LH surge timing after beginning of the dark phase (16h00). (g) LH surge amplitude before (PRE), during (EXPO) and after (POST) adult BPA exposure (BPA-25ng: n=5; BPA-5mg: n=5). (h) GnRH interpulse interval *in vitro* using hypothalamic explants obtained on PND 106, i.e. 24h after the last administration of BPA or corn oil (CTL) in adult female rats (n=4/group). * $p<0.05$, ** $p<0.01$ vs CTL. Data was analysed using a two-way ANOVA and represented as mean $\pm$ SEM.

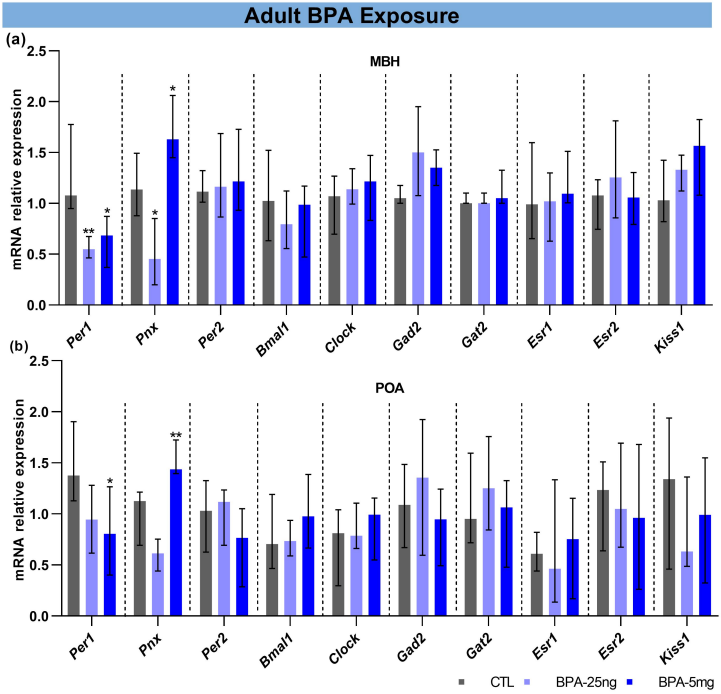


Figure 7.4. Effects of adult (P90 to 105) exposure to BPA (25 ng/kg/d or 5 mg/kg/d) or corn oil (CTL) on relative gene expression in the mediobasal hypothalamic (a) or median preoptic (b). Females in diestrus 24h after the last BPA dose were analysed for relative mRNA expression of *Per1*, *Pnx*, *Per2*, *Bmal1*, *Clock*, *Gad2*, *Gat2*, *Esr1*, *Esr2* and *Kiss1* (CTL: n = 5; BPA-25 ng: n = 6; BPA-5 mg: n = 5). Data was analysed using Mann-Whitney test and represented as median and IQR.

We carried out qPCR analysis of the estrogen receptors *Esr1* and *Esr2*; *Kiss1*, the clock genes *Per1*, *Per2* and *Clock* and the novel peptide *Pnx* in the MBH (Fig.7.4.a) and PoA (Fig.7.4.b) of adult control and BPA exposed females. Genes involved in GABAergic transmission were studied as well because they had been previously shown to be sensitive to neonatal exposure to a low and high dose of BPA (Franssen et al., 2016). MBH and POA were dissected in females in diestrus 24h after the last day of exposure. In the MBH, *Per1* was found to be significantly decreased after exposure to the high ($U = 0.0$, $z = 3.2$, $p < 0.05$, $r = 1.2$) and the low BPA dose ($U = 2.5$, $z = 3.0$, $p < 0.05$, $r = 1.2$). *Pnx* was significantly increased after exposure to the high BPA dose and decreased after exposure to the low BPA dose both in the PoA and MBH. Relative mRNA expression of *Esr1*, *Esr2*, *Kiss1*, *Gad2* and *Gat2* was not significantly affected by BPA in the MBH or the PoA.

7.4 Discussion

In the present study, we provide the first evidence that adult exposure to a low environmentally relevant dose of BPA, in the range of nanograms, disrupts the preovulatory LH surge and leads to abnormal estrous cycle and folliculogenesis. Such disruption is reversible after adult exposure to BPA whereas it persists into adulthood following neonatal exposure, indicating a disruption of ovarian programming. Few studies have directly compared the windows of sensitivity to BPA. Nikaido and coworkers have used BPA during and after the organizational period of sex steroids for reproduction in female mice (Nikaido et al., 2005b; Nikaido et al., 2004). CD-1 female mice were exposed to 10 mg/kg/d of BPA for 4 days either prenatally during the last week of gestation or prepubertally starting at PND 15. In both conditions, a reduced presence of corpora lutea was observed at 4 weeks of age, by the time of vaginal opening. This effect had disappeared at 8 and 24 weeks of age. These data indicate some reversibility of BPA effects on luteinisation following exposure during and after the organizational fetal window. Though we did not study ovarian histology close after the time of vaginal opening, we report here that the time from vaginal opening to the first complete estrous cycle is markedly increased after neonatal exposure to BPA. This is consistent with a reduced likelihood of ovulation and corpora lutea formation. Also, the presence of corpora lutea by 18 weeks of age is not reduced after neonatal exposure in the present study, in agreement with Nikaido's findings. The importance of the selected endpoints is emphasized by our data since the reduced representation of primordial follicles appears here to be the major expression of disrupted ovarian organization.

While the alterations of estrous cyclicity persist after neonatal exposure, they appear to be transient during adult exposure to BPA. During two weeks of adult exposure to a very low or high dose of BPA, altered estrous cyclicity occurs together with disruption of the late stages of folliculogenesis (antral follicles and corpora lutea). Importantly, all these effects appear to have disappeared one month after stopping the exposure to BPA.

Both neonatal and adult exposure lead to alterations characterized by a decrease in the percentage of time spent in proestrus and an increase in the time

spent in diestrus. Wang et al. reported that proestrus was reduced and diestrus increased in 3 month-old mice after fetal exposure to a relatively low dose of BPA (500 ng/kg)(Wang, Hafner, and Flaws, 2014), in agreement with our findings after neonatal exposure of rats to 25 ng/kg. In our study, as the time spent in estrus was not affected by BPA, we hypothesized that the timing of ovulation during proestrus could be affected. To verify this hypothesis, we have measured the LH surge using serial blood sampling on the day of the expected proestrus and our results showed a systematic delay of the LH surge caused by both BPA doses together with a decrease in LH surge amplitude. Gestational exposures to much higher concentrations of BPA in sheep has been previously shown to lead to a dampened (Savabieasfahani et al., 2006) and slightly delayed LH surge (Veiga-Lopez et al., 2013). Other studies have reported decreased amplitude of the LH surge after gestational or prepubertal exposure to p-tert-octylphenol (Herath et al., 2001), perfluorooctanesulfonic acid (Feng et al., 2015), atrazine (Foradori et al., 2011) or polychlorobyphenyls (Steinberg et al., 2008). However, our study is the first one showing such a systematic delay during adult exposure to a low dose of BPA.

Prenatal exposure to BPA has been shown to modify hypothalamic gene expression and behavior in mice and rats (Wolstenholme et al., 2012; Arambula et al., 2016), supporting central mechanisms for BPA effects. We also found that GABA neurotransmission was involved in the neonatal effects of BPA on the neuroendocrine control of GnRH secretion (Franssen et al., 2016). In our previous studies using a model of pulsatile GnRH secretion by hypothalamic explants *ex vivo*, we have shown that neonatal exposure to BPA results in opposing effects on the GnRH interpulse interval depending on the dose (Franssen et al., 2016). In the present study, adult exposure also leads to some opposing central effects of BPA on the GnRH interpulse interval during diestrus studied ex vivo. While a low BPA dose slightly increases the GnRH interpulse interval, the high BPA dose results in a decrease of the interpulse interval. While adult exposure to BPA results in the same pattern of change in GnRH secretion than neonatal exposure, the effect is quantitatively less important. However, both doses significantly disrupted the LH surge, which brings more evidence regarding the neuroendocrine disruption of ovulation caused by BPA. Additionally, Veiga-Lopez et al., 2013 have shown that

the preovulatory estradiol rise in prenatal BPA-treated female sheep was similar to that of controls which indicates that the ovarian signal is normal and the defect involves the neuroendocrine control of the LH surge generation. We have shown here that the expression of phoenixin (*Pnx*), a newly discovered hypothalamic peptide linked to reproduction, is sensitive to adult exposure to BPA. *Pnx* is thought to be involved in the preovulatory LH surge through stimulation of GnRH and kisspeptin release and has been recently shown to be sensitive to BPA (McIlwraith, Loganathan, and Belsham, 2018; Treen, Luo, and Belsham, 2016). Knock-down of *Pnx* receptor (*GPR173*) using intracerebroventricular injection of siRNA doubled the length of the estrous cycle in female rats and eliminated the *Pnx*-induced increase in plasma LH (Steyn et al., 2013; Yosten et al., 2013). Therefore, the decrease in *Pnx* expression that we observed indicates that this crucial regulator of GnRH and kisspeptin could be involved in the disruption of ovulation caused by BPA. Conversely, the high BPA dose led to an increase in *Pnx* expression in the MBH and POA. Such dose-related opposite effects on hypothalamic genes have been previously reported by our team after neonatal exposure to BPA (Franssen et al., 2016). Because higher concentrations of BPA have been shown to decrease *Gpr173* expression in immortalized hypothalamic neurons, the increased expression of *Pnx* after the high BPA dose could be interpreted as reactional.

The AVPV nucleus is a region critical for the occurrence of the LH surge and is known to be sensitive to endocrine disruptors. Decreased *Kiss1* and *Esr1* mRNA expression in the AVPV was observed on postnatal day 10 after exposure to BPA (Cao et al., 2011) while adult exposure might increase *Kiss1* mRNA expression in the AVPV (Wang, 2014). Altogether, a decreased hypothalamic-pituitary sensitivity, caused by a failure of the AVPV to respond to peripheral signals could explained the impaired LH surge caused by BPA. However, mRNA levels of estrogen receptors and Kisspeptin were not affected neither in the mPoA nor the mediobasal hypothalamus in our model. Further studies should look at specific AVPV expression. The occurrence of the preovulatory LH surge depends on the master circadian clock within the suprachiasmatic nucleus together with rising ovarian estrogen levels. The clock genes *Per1* and *Bmal1* in the AVPV play a critical role as integrator of ovarian and circadian signals to time the LH surge (Everett

and Sawyer, 1950; Smarr, Gile, and Iglesia, 2013) and appear to be sensitive to endocrine disruption (Loganathan et al., 2019). Recently, Loganathan et al. (2019) showed that BPA was able to alter *Bmal1* and *Per2* expression in immortalized hypothalamic neurons. Our data indicates that *Per1* expression in the hypothalamus is sensitive to adult exposure to BPA and suggests that clock genes could be the central link explaining the effect of BPA on the LH surge timing. Further studies will need to look at *Per1* expression in the AVPV throughout the afternoon of the proestrus.

Alternatively, disruption of the LH surge by BPA could be explained through indirect effect on energy balance and homeostasis. Indeed, NPY and POMC are known to be involved in the regulation of the LH surge in rats (Kalra, Bonavera, and Kalra, 1995; Pillon et al., 2003; Sahu, Crowley, and Kalra, 1995) and to be sensitive to exposure to BPA. Gestational exposure increases *Npy* and *Agrp* and decreases *Pomc* hypothalamic expression in male rats (Desai et al., 2018). Female mice exposed *in utero* and during lactation show reduced proopiomelanocortin fiber innervation into the AVPV nucleus and increased adiposity and leptin serum levels (Mackay et al., 2013). Interestingly, recent *in vitro* data using hypothalamic cell lines and primary cultures indicate that Bisphenol A requires *Bmal1*, a clock gene known to increase *Npy* expression in hypothalamic neurons (Loganathan et al., 2019). Whether the effects of BPA on the LH surge are mediated by targeted changes in components of the energy balance remain unknown.

The exposure to BPA during gestation or neonatal life can affect ovarian structure and function (Diamanti-kandarakis, Palioura, and Kandaraki, 2012). Fetal exposure to BPA was shown to increase cells in germ cell nests and to reduce primordial follicles (Wang, 2014). The neonatal period is critical for ovarian differentiation since formation of primordial follicles are not completed until PND 3-4 and initial recruitment takes place during neonatal life. Thus, disturbances in early stages of folliculogenesis can also occur after neonatal exposure to BPA (Rodriguez et al., 2010). Our findings indicate that reduced pools of primordial follicles or antral follicles could reflect insults during or after development, respectively. In the CLARITY-BPA study (Patel et al., 2017), exposure to BPA 2.5 and 250 μg/kg/d from GD 6 to PND 21 resulted in reduced primordial, primary and preantral follicles at PND 21. However, irrespective of stopping exposure to

BPA at PND 21 or continuing till the end of experiment, there were no longer any alteration of folliculogenesis at 3 and 6 months of age. By 1 year of age, cystic follicles were found only after exposure to 25 mg/kg/d of BPA till PND 21. These data indicate possible developmental effects of BPA though they appear to be transient and reversible, even during sustained exposure. Among the factors possibly accounting for discrepancies between the CLARITY-BPA study and the present one, the dose of BPA, the route of administration and the age window of exposure could play some role as well as differences in rat strain (Wistar versus Sprague-Dawley). In some studies, the number of primary follicles was either reduced (Santamaria et al., 2016) or increased (Gámez et al., 2015) after early postnatal exposure to BPA in the μg/kg dose range. Interestingly, fetal exposure of mice (from GD 6 to birth) to a lower dose of BPA (500 ng/kg/d) than in the CLARITY-BPA study resulted in reduced presence of antral follicles at PND 21 (Berger et al., 2016) and at 3 months of age (Mahalingam et al., 2017). These findings are consistent with ours regarding persistent effects in adulthood after early life exposure though those authors did not find any reduction in primordial follicles (Mahalingam et al., 2017). In similar conditions, however, the number of primordial follicles was reduced on PND 4, indicating that both the age at exposure and the age at evaluation matter (Wang, 2014). Taken together, those data point to the requirement of additional studies involving postnatal and sustained exposure to very low doses of BPA in the ng/kg range.

Human exposure to BPA is sustained throughout life and provides the rationale for lifelong exposure as done in the CLARITY-BPA study (National Toxicology Program, 2018). We elected to expose the animals to BPA transiently for 2 weeks because a transient exposure was required to evaluate whether effects persisting into adulthood could result from neonatal exposure. Likewise, transient exposure in adulthood was necessary to study possible reversibility of the effects after exposure in conditions comparable with those used neonatally. In contrast to the CLARITY-BPA study in rats and other studies in mice, our conditions did not include fetal exposure (Berger et al., 2016; Mahalingam et al., 2017; Wang, 2014; Brannick et al., 2012). The subcutaneous route of administration was indispensable for reliable administration of BPA to neonatal rats, particularly using such a very low dose as discussed previously (Franssen et al., 2016).

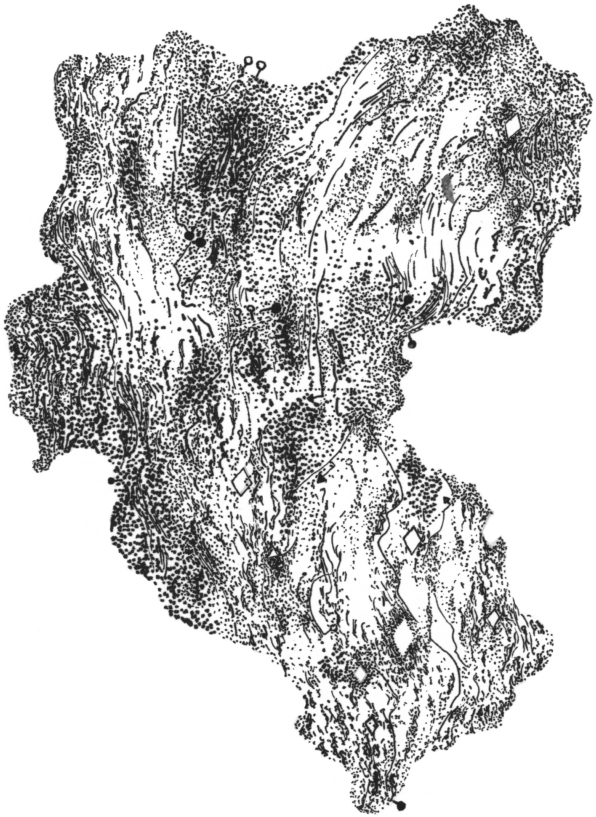
This required control for contamination by other BPA sources through the use of low-phytoestrogen pellets, glass-bottles and BPA-free cages. The oral route could not allow reliable administration of BPA doses in the range of ng/kg. This possibly explains that such very low doses were not used in the CLARITY-BPA study (National Toxicology Program, 2018). While the oral route is consistent with the human conditions of exposure, oral gavage as done in the CLARITY-BPA study can account for confounding factors such as stress and bypass of oral absorption (Vandenberg et al., 2014a). Comparable serum levels of BPA and UDP-glucuronosyltransferase, the enzyme that conjugates BPA have been reported after oral and subcutaneous administration neonatally (Taylor, Welshons, and Vom Saal, 2008). However, in another more extensive study in neonatal mice, the systemic levels of free BPA were found to be 3-4 times higher after subcutaneous injection than after oral administration (Draganov et al., 2015). Assuming that the pups in our study would have been exposed to BPA levels 4 times higher than using the oral route, such levels (equivalent to 100 ng/kg orally) would still be consistent with human exposure and 25 times less than the lowest dose used in the CLARITY-BPA study (National Toxicology Program, 2018). For consistency and to ensure precision in the low levels administered, we also used the subcutaneous route of administration in the adult females. The reversibility of the effects of the low BPA dose after resumption of control conditions supports the evidence that the effects of the very low dose are unlikely resulting from a contaminant since all the management conditions except BPA (vehicle, food, drink and cages) were identical in the control and treatment settings. That very low dose is far below the No-Observed-Adverse-Effects Level (NOAEL) and below the EFSA "safe dose". It represent half the average exposure of the general population (Vandenberg et al., 2007). Following neonatal or adult exposure to BPA, the ovulatory cycle and folliculogenesis are impaired and the effects are similar using a very low dose of BPA or a high dose in the range of milligrams. Altogether, the present study suggests that the effect of BPA on the ovaries is more dependent on the period of exposure in life than the dose of BPA though only two doses were studied and the effects of intermediate doses warrant further studies.

7.5 Conclusions

In conclusion, we show that both adult and neonatal exposure to a very low dose of BPA in the range of nanograms can result in alteration of estrous cyclicity and folliculogenesis. Similar alterations are observed using a high dose of BPA. Neonatal exposure leads to effects occurring after exposure and persisting on the long-term suggesting that BPA is able to reprogram the reproductive axis at early stages, particularly by affecting the early follicular development. By contrast, adult exposure to BPA causes effects to occur transiently during exposure since normal cyclicity and folliculogenesis are restored within one month after resuming control conditions. Moreover, estrous cyclicity during adulthood seems to be altered by central mechanisms involving the disruption of the LH surge. Our findings imply that when further evaluating BPA adverse effects on the female reproductive axis, very low doses in the range of average environmental exposure should be used with inclusion of the critical neonatal period and addressing both neuroendocrine and ovarian endpoints.

EXPERIMENTAL STUDY

Multi- and transgenerational disruption of maternal behavior and female puberty by EDC mixture exposure



8 Experimental study two

Multi- and transgenerational disruption of maternal behavior and female puberty by EDC mixture exposure

Contents

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Abstract

Female reproductive development and maternal behavior are two intertwined phenotypes centrally controlled by the hypothalamus. Endocrine disrupting chemicals (EDCs) can alter these processes especially when animals are exposed during development. We propose the concept that developmental exposure to a low environmentally relevant dose of EDC mixture induces a transgenerational alteration of female rat pubertal timing and ovarian physiology throughout epigenetic reprogramming of hypothalamic *Kiss1*, *Esr1* and *Oxt1* loci. Such exposure also caused a multigenerational reduction of maternal behavior induced by the loss in hypothalamic dopaminergic signaling. Our results identify the hypothalamic Polycomb Group of epigenetic repressors as actors of this mechanism of transgenerational reproductive disruption. Using a cross-fostering approach, we identified that while the reduction in maternal phenotype was normalized in EDC exposed pups raised by unexposed dams, no reversal of the pubertal phenotype was achieved, suggesting a germline transmission of the reproductive phenotype.

8.1 Introduction

Endocrine Disrupting Chemicals (EDCs) impact populations as much as individuals given their environmental ubiquity (Gore et al., 2015). Among 85,000 chemicals in use, 1,000 have been identified as having the ability to disrupt normal endocrine function (Bergman et al., 2013). Developmental exposure to EDCs has been associated with increased risk of genital malformations, hypofertility and testis cancer in human and rodent males, increased risk of breast cancer and metabolic syndrome in adulthood, as well as alterations of pubertal timing and ovarian function (Demeneix and SLAMA, 2019). Fetal development as well as childhood and adolescence are known windows of sensitivity to chemical exposure, which can alter the developmental trajectory and lead to long-lasting dysfunction (Gore et al., 2015; Parent et al., 2016). In addition to the direct effects of EDCs on individuals, studies have revealed that early exposure affects the health of subsequent generations through epigenetic (transgenerational) mechanisms that alter the germ line methylome, affecting pubertal timing, ovarian follicle development, stress responsiveness and mate preference in subsequent generations (Anway et al., 2005; Manikkam et al., 2012a). Alternatively, a non-genetic transmission of parental traits has been shown to propagate multigenerationally (Champagne and Meaney, 2006; Champagne et al., 2001). Maternal care affects offspring development from early postnatal life, inducing somatic changes that lead to the propagation of the same behavioral phenotype such as increased stress responsiveness or low licking and grooming behavior (Mitchell et al., 2016).

In mammals, sexual maturation is driven by increased pulsatile secretion of hypothalamic gonadotropin-releasing hormone (GnRH) released into the portal vasculature that feeds into the pituitary gland, ultimately increasing pulsatile release of luteinizing hormone (LH) from pituitary gonadotrophs into the peripheral circulation, inducing ovarian steroidogenesis and ovulation (Ojeda and Skinner, 2006). During the prepubertal period, the secretory activity of GnRH neurons is under predominant trans-synaptic inhibitory control provided by GABAergic, Opiatergic and RFamide inputs into the GnRH neuronal network (Ojeda, Lomniczi, and Sandau, 2008). At puberty this inhibition is lifted while a concomitant increase in excitatory inputs to the GnRH network is provided by

Glutamatergic and Kisspeptidergic neurons (Terasawa et al., 2018; Clarkson and Herbison, 2006a). Recent evidence suggests that this trans-synaptic regulatory mechanism is controlled by a molecular switch that regulates the timing of puberty. This epigenetic switch coordinates the transcriptional activity of arcuate nucleus (ARC) kisspeptin neurons implicated in stimulating GnRH release (Toro et al., 2018; Lomniczi et al., 2013b). Before puberty, the transcriptional activity of *Kiss1*, the gene encoding for kisspeptins, is downregulated by members of the Polycomb Group (PcG) of transcriptional repressors, by catalyzing the trimethylation of histone 3 at lysine 27 (H3K27me3), a histone mark associated with gene silencing (Lomniczi et al., 2013b). As puberty approaches, the PcG is evicted from the Kiss1 promoter and the Trithorax Group (TrxG) of epigenetic activators is recruited, resulting in increased histone methylation at lysine 4 (H3K4me3) and acetylation at lysine 27 (H3K27ac) to the *Kiss1* promoter/enhancer regions respectively, leading to an increase in *Kiss1* mRNA transcription (Toro et al., 2018).

Here we propose the concept that developmental exposure to an EDC mixture induces a transgenerational alteration of female pubertal timing and a multigenerational decrease in maternal behavior. In the current study, female rats (F0) were exposed to a mixture of 13 of the most prevalent EDCs present in the human body at relevant exposure concentrations from 2 weeks before gestation until the end of lactation. The four subsequent generations were evaluated for sexual maturation and maternal behavior. Our data show that gestational and lactational exposure to an environmentally relevant EDC mixture alters maternal behavior in F1 through F3 generations and transgenerationally affects sexual development by epigenetic reprogramming of the hypothalamus. While F2, F3 and F4 females display delayed puberty and abnormal estrous cycles, such changes are not detected in F1 *in vitro* and lactationally exposed animals. As the phenotype appears at the F2 generation, these data suggest that germline exposure is required to disrupt reproductive development. These phenotypes are associated with alterations in both transcriptional and histone posttranslational modifications of hypothalamic genes involved in dopamine signaling and GnRH neuron pulsatility control. By using a cross-fostering paradigm, we show that the reproductive alterations are transmitted through a germline-dependent mechanism, while the alteration in maternal behavior is, at least in part, produced by the direct exposure of the fetus

to the EDC mixture.

8.2 Methods

Animals

Adult Female Wistar rats purchased from the animal facility of the University of Liège were housed individually in standardized conditions (12h inverted dark/light phase, 22.8°C and food and water *ad libitum*). All animals were raised in EDC-free cages (Polypropylene cages, Ref 1291H006, Tecnilab, Netherlands) and fed EDC- and phytoestrogen-free chow (V135 R/Z low phytoestrogen pellets, SSNIFF Diet, Netherlands). Water was supplied in glass bottles. All experiments were carried out with the approval of the Belgian Ministry of Agriculture and the Ethics Committee at the University of Liege.

Chemicals

The endocrine disrupting chemicals di-n-butylphthalate (DBP) (purity >99.0 %, 84-74-2), di-(2-ethylhexyl)phthalate (DEHP) (purity >99.5 %, 117-81-7), vinclozolin (purity >99.5 %, 50471-44-8), prochloraz (purity >98.5 %, 67747-09-5), procymidone (purity >99.5 %, 32809-16-8), linuron (purity >99.0 %, 330-55-2), epoxiconazole (purity >99.0 %, 106325-08-8), 2-ethylhexyl 4-methoxycinnamate (OMC, EHMC) (purity >98.0 %, 5466-77-3), dichlorodiphenyldichloroethylene (p,p'-DDE) (purity >98.5 %, 72-55-9), 4-methyl-benzylidene camphor (4-MBC)

Substance	Function	AA/E	Dose	NOAEL	LOAEL
			(µg/kg/d)	*(mg/kg/d)*	
Di-n-butyl-phthalate (DBP)	Plasticizers	AA	10	50	100
Di-(2-ethylexyl)-phtalate (DEHP)		AA	20	3	10
Bisphenol A (BPA)		AA / E	1,5	5	1,2
Vinclozolin	Dicarboximide fungicide	AA	9	5	10
Procymidon		AA	15	10	25
Prochloraz	Imdazole fungicide	AA	14	5	10
Linuron	Urea-based herbicide	AA	0,6	25	50
Epoxinaxole	Triazole fungicide	AA	10	15	-
4-Methyl-benzylidene camphor (4-MBC)	UV-filter	E	60	0,7	7
Octyl methoxycinnamate		E	120	-	500
p'p'-DDE	Metabolite of insecticide DDT	AA / E	1	-	10
Butyl paraben (BP)	Antifungal and antibacterial preservative	AA	60	-	100
Acetaminophen	Analgesic & antipyretic	AA	800	-	350

Table 8.1. Features of the 13 chemicals included in the EDC mixture. AA: antiandrogenic, E: estrogenic, NOAEL: non-observed adverse effect level, LOAEL: low-observed adverse effect level.

(purity >98.0 %, 36861-47-9) and butylparaben (purity >99.0 %, 94-26-8) were purchased from AccuStandard. Bisphenol A (purity >99.5 %, 80-05-7), and paracetamol (purity >99.0 %, 103-90-2) and corn oil (as a control vehicle) were obtained from Sigma-Aldrich. EDC compounds were dissolved in corn oil in order to obtain the final concentration showed in Table 8.1.

Experimental design

After habituation to the animal care facility, F0 generation females were mated for two weeks with a wild-type male in order to generate the F1 generation. F0 females were exposed to an EDC mixture or corn oil (vehicle) from two weeks before gestation to the last day of lactation (Fig.8.1). Daily exposure was done by injecting 50μl of EDC mixture or corn oil in a waffle and allowing them to eat it. Verification was systematically done after 10 minutes of exposure. Females were randomly assigned to treatment. At postnatal day (P) 70, *in utero* EDC mixture exposed and control females from the F1 generation were mated with a wild-type male to generate the F2 generation. Similar procedure was done to generate the F3 and F4 generation. Pups from every generation were homogenized by sex-ratio and litter size and followed for developmental weight until weaning (P21). From weaning to P70, control females were paired with a female EDC and housed together under the same conditions. Behavioral and reproductive data were obtained from two independent non-related cohorts of animals.

Cross-fostering

To distinguish between germ-cell versus experience-based phenotype transmission, a cross-fostering paradigm was used. In order to avoid an effect of cross-fostering, a maximum of two pups per litter were cross-fostered in pups from F1 female dams as followed. F1 generation control dams raised either EDC mixture exposed pups (CE) or control pups from another dam (CC). F1 generation EDC mixture exposed dams raised either EDC mixture exposed pups from another dam (EE) or control pups (EC). Cross-fostering was carried out within the first 24h after delivery and a 1mm tail incision was done in order to distinguish cross-fostered pups.

Maternal Behavior

To assess the effect of an EDC mixture exposure throughout generations on ma-

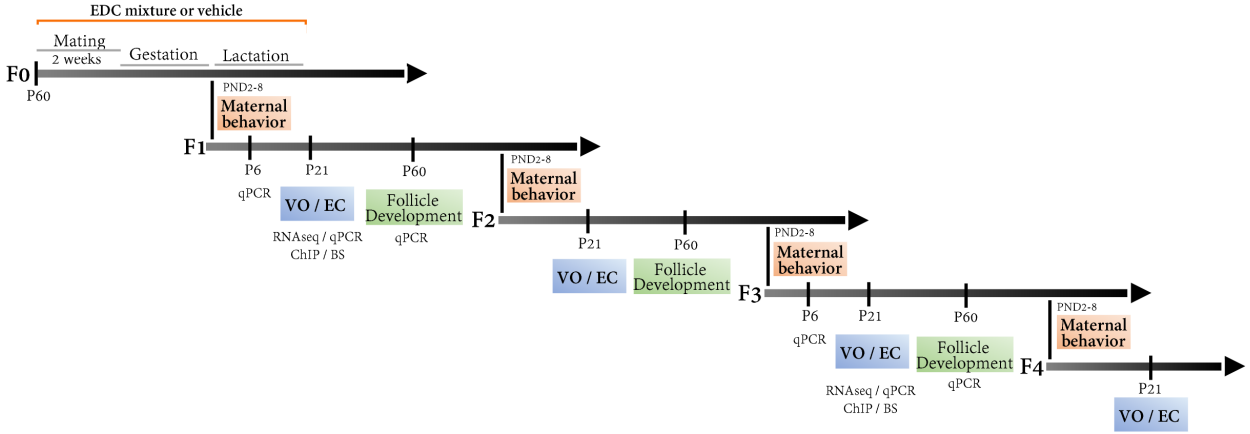


Figure 8.1. Multi- and transgenerational design of an EDC mixture exposure on maternal behavior and sexual maturation. Female rats (F0) were exposed to a mixture of 13 of the most prevalent EDCs present in the human body at relevant exposure concentrations from 2 weeks before gestation until the end of lactation. The four subsequent generations were evaluated for sexual maturation (vaginal opening, GnRH interval interpulse, estrous cycle and folliculogenesis) and maternal behavior (from P2 to P8). Massive parallel RNA sequencing was carried out using mediobasal hypothalamic (MBH) explants from the F1 and F3 generation to decipher direct (F1) versus transgenerational (F3) target genes of the EDC mixture exposure, followed by qPCR validation at three time points, P6, 21 and 60. Target genes were studied for histone posttranslational modifications and DNA methylation using chromatin immunoprecipitation (ChIP) and bisulfite sequencing, respectively. V.O: vaginal opening, E.C: estrous cycle.

ternal behavior, a set of maternal behaviors were quantified in EDC mixture and control lactating females from the F0 to the F3 generation. Additionally, we have quantified maternal behavior in F1 dams (EDC mixture exposed or control dam with either control or EDC mixture exposed pups from another dam) with cross-fostered pups and in F2 cross-fostered lactating females (EDC mixture exposed or non-exposed females raised by an EDC mixture exposed or control dam). Maternal behavior was recorded with an infrared camera (Bell and Howell, DNV16HDZ-BK) from lactational day 2 to 8 for 1 hour during the dark phase. Randomization was done in order to avoid recording every female at the same hour of the day. A set of in-nest behaviors (retrieval, mouthing, licking/grooming, arched-back/blanked/passive nursing and nest building) and off-nest behaviors (eating/drinking, grooming, active or resting alone) were quantified as reviewed in Lonstein et al., 2015 by an experimenter blind to condition.

Pubertal onset and estrous cyclicity

To assess the effect of an EDC mixture exposure throughout generation on sexual maturation, females from the F1 to the F4 generation were followed for vaginal opening and estrous cyclicity as described previously (Toro et al., 2018; Franssen et al., 2014). Briefly, from P25, females were daily inspected for vaginal opening by two experimenters. From the day of vaginal opening to P70 estrous cycle was evaluated with vaginal smears that were taken every day during the morning before the lights off at 4pm. Regular cycles were defined as a sequence of diestrus 1, diestrus 2, proestrus and estrus in 4 consecutive days (Goldman, Murr, and Cooper, 2007). The percentage of females having a regular cycle and the time spent in every stage of the cycle were calculated from week 6 to week 10 of age.

Ovarian histology and uterus weight

To assess the effect of an EDC mixture exposure on folliculogenesis throughout generations, ovaries from F1 to F3 generation females at P70 were removed for histological quantification of follicle development. After removal, ovaries were weighted together with the uterus and fixed overnight in 4% paraformaldehyde, dehydrated in 70% EtOH and paraffin-embedded. Histological analysis was done in 8-μm coronal sections (microtome RM2245, Leica), after deparafinization and stain with hematoxylin and eosin. For quantification, every other section throughout the whole ovary were digitalized using an automated digital mi-

croscopy system DotSlide (Olympus, BX51TF, Aartselaar, Belgium). Dotslide images taken at a magnification of 10x, which were in a proprietary format were converted into a standard TIFF format and 3-fold decimated, easier to handle. Thereafter, follicles at every stage of folliculogenesis (primordial, primary, secondary, antral and atretic), cystic follicles and corpora lutea were manually quantified avoiding double-counting by an experimenter blindness to treatment with Aperio ImageScope v12.3.2.8013 software (SCR014311, Leica Biosystems). Total ovarian volume was automatically calculated using an original program developed using the image analysis toolbox of the MatLab (SCR001622, 2016a, The Mathworks Inc., Natick, MA, USA) software. The follicles were classified according to well-established criteria (Hirshfield and Midgley Jr., 1978; Peters, 1969). Double counting of late-stage follicles was avoided by digitally marking each follicle throughout the consecutive images. Each follicle was counted once whenever the oocyte was present. As analysis were done in every other section, we apply a two-fold correction factor for quantification of early stage follicles (primordial and primary follicles) to compensate sections not analysed. Measurements are expressed as number of follicles or corpora lutea per volume (mm^3).

Hypothalamic explants incubation and GnRH assay

To assess the effect of an EDC mixture exposure on juvenile GnRH frequency, GnRH interpulse interval was measured using a hypothalamic explants incubation system followed by a GnRH assay from prepubertal females of the F1 and F3 generation, as described previously (Bourguignon and Franchimont, 1984b). Briefly, after decapitation, brain was dissected by performing two sagital incisions along the lateral hypothalamic sulci and two transversal incisions of 2mm ahead from the anterior boundaries of the optic chiasm and along the caudal margin of the mammillary bodies. Once dissected, MBH and PoA explants were transferred into an individual chamber, in a static incubator, submerged in MEM. Incubation medium was then collected and renewed every 7.5 min for a period of 4 hours. The GnRH released into the incubation medium was measured in duplicate using a radioimmunoassay method with intra and inter-assay coefficients of variation of 14 and 18% respectively. The highly specific CR11-B81 (AB2687904) rabbit anti-GnRH antiserum (final dilution 1:80,000) was kindly provided by Dr. V.D. Ramirez (Urbana, IL) (Dluzen and Ramirez, 1981). Data below the limit of de-

tection (5 pg/7.5-min fraction) were assigned that value.

DNA and RNA extraction, reverse transcription and qPCR

Expression of genes involved in the hypothalamic control of puberty, reproduction and maternal behavior were studied by quantitative PCR (qPCR) analysis using the half of MBH and mPoA explants from females exposed and non-exposed to an EDC mixture from the F1 and F3 generations at different time points (P6, P21 and P60). After decapitation, the mPoA and the MBH were rapidly dissected as described in the previous section. Additionally, MBH and PoA were divided in two by sectioning along the interhemispheric fissure. MBH fragments contain the entire arcuate nucleus. Total RNA and DNA were extracted from the half-MBH and half-mPoA tissue using All Prep DNA/RNA Mini kit (Qiagen, Germantown, MD) following the manufacturer's instructions. Five hundred ng of RNA for each sample were reverse transcribed using the Transcriptor first strand cDNA synthesis kit (Roche, Germany). For real-time quantitative PCR reactions, the cDNA of our samples were diluted 10 fold and 4μl were added to a mix of 5μl FastStart Universal SYBR Green Master (Roche, Germany), 0.4μl of nuclease-free water and 0.3μl of forward and reverse primer (see primer sequences in appendix E.1). The samples were run in triplicate using a LightCycler 480 thermocycler (Roche, Germany). Ct values were obtained from each individual amplification curve and the average Ct was calculated for each target gene in each sample. Quantification of relative gene expression was performed using the $\Delta\Delta$Ct method implemented with the Pfaffl equation which takes into account reaction efficiency depending on primers(Pfaffl, 2001). All assays had efficiencies between 1.9 and 2.1. β-actin was used as housekeeping gene.

Bisulfite sequencing

Genomic DNA was Bisulfite-converted (BC) using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. The BC DNA was used as input material for PCR amplification followed by library preparation and deep sequencing. Primers were designed to amplify a 302bp region of the rat *Th* promoter, including exon 1 (-139 to $+164$ bp from the *Th* TSS) (see primer sequences in appendix E.1). Amplification was carried out on a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) with 20ng of BC DNA per reaction. The amplification conditions were: 40 cycles of 94°C for 30sec, 55°C

for 30sec and 72°C for 1min sequencing libraries were prepared using the NETflex DNA Sequencing Kit (BIOO Scientific, Austin, TX) according to manufacturer's instructions. The libraries were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and were normalized to 2nM with 10 mM Tris-HCl. The libraries were then pooled and sequenced on a MiSeq (Illumina, Inc. San Diego, CA) by the Molecular & Cellular Biology Core, (ONPRC, Beaverton, OR) to generate 250 base, paired-end reads. The reads were trimmed using Trim Galore and aligned to the reference genome using Bismark. Alignment data was converted to CpG methylation rate using the Bismark methylation extractor and custom scripts.

Immunohistochemistry

To assess the expression of proteins related to maternal behavior, immunoreactivity of TH was measured in EDC mixture exposed and non-exposed females from the F1 generation at P21. TH-ir was measured in the substantia nigra (SN), the ventral tegmental area (VTA) and the median preoptic area (mPOA). Females were anesthetized with pentobarbital sodium ($50 mg kg-1$, i.p.) and sequentially perfused with PBS and 4% paraformaldehyde. Brains were subsequently removed and post-fixed in 4% paraformaldehyde at 4°C overnight. Therefore, coronal sections (30μm) were cut on a vibratome and used for immunohistofluorescence.

For immunohistochemistry, sections were incubated in PBS and blocked with 10% donkey serum for 1h at room temperature, and incubated overnight with the primary antibody anti-TH (Th-mouse, 22941, ImmunoStar, Hudson, USA, 1:1000) for 24h at room temperature or 4°C. Thereafter, the corresponding fluorophore-conjugated secondary antibodies were incubated for 2h at room temperature. The high-resolution Zeiss microscope LSM880 implemented with the fast Airyscan detector was used for visualization. Three slides per animal per region of interest (ventral midbrain sections containing the SN and VTA or median preoptic area sections) were used for quantification. An observer blind to condition outlined each region of interest for nuclei-specific analysis. Region area and number of immunoreative cells were automatically quantified using Imaris 9.3. Statistics were performed on the total cell count per animal per region across slice sections.

Massively parallel RNA sequencing (RNAseq)

Poly-A mRNA was purified from 1μg total RNA using NETFLEX poly-A beads (Perking-Elmer, Austin, TX) followed by library preparation using the NETFLEX Rapid Directional RNA-seq Kit 2.0 (Perking-Elmer). In short, after fragmentation with divalent cations and heat, mRNA was used as template for reverse transcription using random hexamer primers. cDNAs were then blunted and 3' end A-tailed to facilitate adaptor ligation. Six-base pair Illumina adaptors were ligated followed by 12 rounds of PCR amplification. Free dNTPs were removed using AMPure XP beads (BeckmanCoulter, Brea, CA). Distribution of DNA sizes in the library was confirmed by Bioanalyzer analysis (Agilent, Santa Clara, CA). Library titer was determined by real-time PCR (Kapa Biosystems, Wilmington, MA) on a Quant Studio 12K Flex Real Time PCR System (ThermoFisher, Waltham, MA). Four samples were sequenced per lane on a HiSeq 4000 (Illumina). Sequencing was done using a single-read 100-cycle protocol. The resulting base call files (.bcl) were converted to standard fastq formatted sequence files using Bcl2Fastq (Illumina). Sequencing quality was assessed using FastQC (Babraham Bioinformatics, Cambridge, UK). The RNAseq procedure was carried out by the Genomics & Cell Characterization Core Facility at the University of Oregon. To determine the differential gene expression values we used the gene-level edgeR analysis package. We performed an initial trimming and adapter removal pass using Trimmomatic. After this reads were aligned to the rn6 build of the rat genome with Bowtie2/Tophat2, and assigned to gene-level genomic features with the Rsubread featureCounts package based on the Ensembl 83 annotation set. Differential expression between experimental groups was analyzed using the generalized linear modeling approaches implemented in edgeR. Lists of differentially expressed genes/transcripts were identified based on significance of pairwise comparison of experimental groups. Gene ontology and enrichment analysis were performed using the database for annotation, visualization and integrated discovery (DAVID).

ChIP assay

To assess activatory and repressive histone modifications at specific gene promoters affected by EDC mixture exposure we performed ChIP assay using extracted chromatin from the hypothalamus of prepubertal rats at P21. As described pre-

viously (Lomniczi et al., 2013b; Mueller et al., 2011), ChIP procedure was carried out by crosslinking tissue in phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (PI, 1 mM phenylmethylsulfonylfluoride, 7 μg ml-1 aprotinin, 0.7 μg ml-1 pepstatin A, and 0.5 μg ml-1 leupeptin), a phosphatase inhibitor cocktail (PhI, 1 mM β-glycerophosphate, 1 mM sodium pyrophosphate, and 1 mM sodium fluoride), and an HDAC inhibitor (20 mM sodium butyrate) at 4°C and 1% formaldehyde for 10 min at room temperature. After two washing steps in PBS, samples were lysed with 200 μl SDS buffer (0.5% SDS, 50 mM Tris-HCl, and 10 mM EDTA) containing protease, phosphatase, and HDAC inhibitors, and sonicated for 45 sec to yield chromatin fragments of approx. 500 base pairs (bp) using the microtip of a Fisher Scientific FB 705 sonicator. Size fragmentation was confirmed by agarose gel electrophoresis. The sonicated chromatin was clarified by centrifugation at 14,000 rpm. for 10 min at 4°C, brought up to 1 ml in Chip Dilution Buffer (16.7 mM TrisHCl, pH 8.1, 150 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, and 0.01% SDS) containing the PI and PhI cocktails, and the HDAC inhibitor described above. The samples were then stored at -80°C for subsequent immunoprecipitation. For this step, chromatin was pre-cleared with Protein A/G beads (Dynabeads, Invitrogen) for 1 h at 4°C. Twenty-five to 50 μl aliquots of chromatin were then incubated with 2-5 μg of the antibodies described in appendix E.2. The complexes were incubated with 25 μl of protein A or G beads solution (Dynabeads) at 4 μC overnight with mild agitation. The next day the beads were washed first with 0.5 ml low-salt wash buffer (20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS), followed by high-salt wash buffer (20 mM Tris-HCl, pH 8.1, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS), LiCl buffer (10 mM Tris-HCl, pH 8.1, 250 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 1 mM EDTA), and finally with TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Thereafter, the immunocomplexes were eluted with 100 μl of 0.1 M NaHCO3 and 1% SDS at 65°C for 45 min. To reverse the crosslinking reaction, we added 4 μl of 5 M NaCl and incubated the samples at 95°C for 30 min. We recovered the DNA using ChIP DNA Clean & Concentrator columns (Zymo Research, Irvine, CA), and stored the resulting material at -80°C before qPCR analysis. All the chemicals mentioned above were purchased from Sigma-Aldrich.

qPCR detection of chromatin immunoprecipitated DNA

Genomic regions of interest were amplified by qPCR. Primer sequences, accession numbers of the genes analyzed as well as the chromosomal position of the 5′−flanking region amplified, using the position of the TSS as the reference point, are shown in appendix E.1. PCR reactions were performed using 1 μl of each immunoprecipitate (IP) or input samples, primer mix (1 μM each primer), and SYBR Green Power Up Master Mix (Thermo Fisher) in a final volume of 10 μl. Input samples consisted of 10% of the chromatin volume used for immunoprecipitation. The thermocycling conditions used were as follows: 95°C for 5 min, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. Data are expressed as % of IP signal/input signal.

Statistics

All statistical analyses were performed using Prism 7.0 software (GraphPad, San Diego, CA). Data was subjected to a normality and an equal variance test and parametric test were used when conditions were accomplished. Parametric test used were one-way or two-way ANOVA followed by Student–Newman–Keuls or Sidak's for multiple comparisons, respectively; or the Student's t-test to compare two groups. When comparing percentages, groups were subjected to an arcsine transformation before statistical analysis to convert the values from a binomial to a normal distribution. Data that did not accomplish normality followed a Mann-Whitney test. When making multiple comparisons α was adjusted by using the Bonferroni correction. The investigator was group blinded in all physiological and molecular determination. Samples size, reported in figure captions, were estimated based in previous studies or based in the calculation of an adequate statistical power. Effect sizes were calculated according Cohen's δ formula (see appendix E.3, E.4 and E.5).The level of statistical significance was a P value < 0.05.

8.3 Results

Exposure to EDC mixture alters pubertal onset, estrous cycle and folliculogenesis throughout generations

Sexual maturation was followed for 4 generations (F1 to F4) of female rats after exposure of F0 females to a mixture of 13 anti-androgenic and estrogenic EDC or corn oil (vehicle) (Fig.8.1). F0 females were exposed orally for 2 weeks before and throughout gestation until weaning. While F1 females, which were directly exposed to EDCs *in utero* (F1-EDC), had normal pubertal timing (Fig.8.2.a) determined by age at vaginal opening; F2, F3 and F4 females had significantly delayed vaginal opening (Fig.8.2.b-d). Maturation of GnRH secretion preceding puberty is characterized by a reduction of GnRH interpulse interval between P15 and P25 in hypothalamic explants incubated individually (Bourguignon and Franchimont, 1984b). While GnRH interpulse interval was not affected in F1-EDC at P20 (Fig.8.2.a), it was significantly increased in F3-EDC females (Fig.8.2.c), suggesting a delayed maturation of GnRH secretion, consistent with a delayed onset of puberty. In addition, estrous cyclicity was disrupted in F2 and F3-EDC females (Fig.8.3.b-c left) with a significant decrease in the proportion of females showing regular cycles, characterized by increased time spent in estrus and reduced time in diestrus. Additionally, F3-EDC females displayed a decreased time spent in proestrus. All these data show globally a sign of subfertility. At P70, ovaries from F1, F2 and F3 females were evaluated for ovarian folliculogenesis. *In utero* exposure to EDCs did not affect follicular development in F1-EDC females (Fig.8.3.a right). In contrast, F2-EDC and F3-EDC females displayed a significant decrease in antral follicles and an increase in atretic follicles (Fig.8.3.b-c right) as compared to controls. Additionally, F2-EDC females showed a significant decrease in the number of primordial follicles (Fig.8.3.b right). Ancestral exposure to the EDC mixture also decreases ovarian weight over time and increases the number of cysts.

Altogether, these results indicate that the EDC mixture exposure did not affect pubertal onset or ovulation in F1 females, a generation directly exposed *in utero* during development, while the next generations (F2, exposed as primordial cells in the ovaries of the F1) and F3 and F4 (ancestrally exposed to EDC)

displayed delayed maturation of GnRH secretion, delayed pubertal onset and abnormal folliculogenesis.

EDC mixture exposure disrupts the epigenetic programming of the hypothalamus

We hypothesized that the delay in GnRH secretion maturation and pubertal onset observed in F3-EDC females could be due to transcriptional and epigenetic disruption of the hypothalamic networks controlling pubertal development. Next, we used massive parallel RNA sequencing to identify gene regulatory pathways altered by EDC exposure in the MBH of F3-EDC females at P21, corresponding to the juvenile phase of prepubertal development. Gene ontology analysis showed that the main downregulated biological processes were: Neurohypophyseal hormone activity, Neuropeptide receptor activity and Dopamine signaling. Some of the downregulated molecular functions affected included: GABA, Dopamine and Glutamate signaling, while xenobiotic processes were upregulated (see supplementary appendix Fig.E.1). qPCR quantification of hypothalamic genes critical for pubertal onset validated the downregulation of *Kiss1*, *Esr1*, and *Oxt* in F3-EDC females at P21 and P60 (Fig.8.4.a). We also identified a set of genes involved in the control of energy balance and reproduction (*Cart*, *Pomc*, *Grin2d*, *Gri2* and *Avp*) and stress responsiveness (*Nr3c1* and *Crh*) that were altered at P21 in F3-EDC females as compared to controls (Fig.8.4.a, see supplementary appendix Fig.E.2). In order to gain insight into the potential contribution of histone modifications to the transcriptional changes caused by EDC exposure, we used ChIP-assay to quantitate the repressive (H3K27me3 and H3K9me3) and activating (H3K4me3, K3K9ac) histone modifications at the promoter region of target genes in the hypothalamus of F3 control and EDC females (Fig.8.4.b, see supplementary appendix Fig.E.3). Transcriptional downregulation caused by EDC mixture was consistently associated with either an increase in repressive histone marks or a decrease in activating histone modifications at the promoter of genes critical for the onset of puberty. We observed an increase in the repressive marks H3K27me3 at the promoter region of the *Kiss1* gene, while a decreased abundance of activating marks H3K4me3 or K3K9ac was observed at *Esr1*, *Kiss1* and *Oxt* (Fig.8.4.b, see supplementary appendix Fig.E.3). No change in histone landscape was detected at the *Cart* promoter while a decrease in the repressive H3K9me3

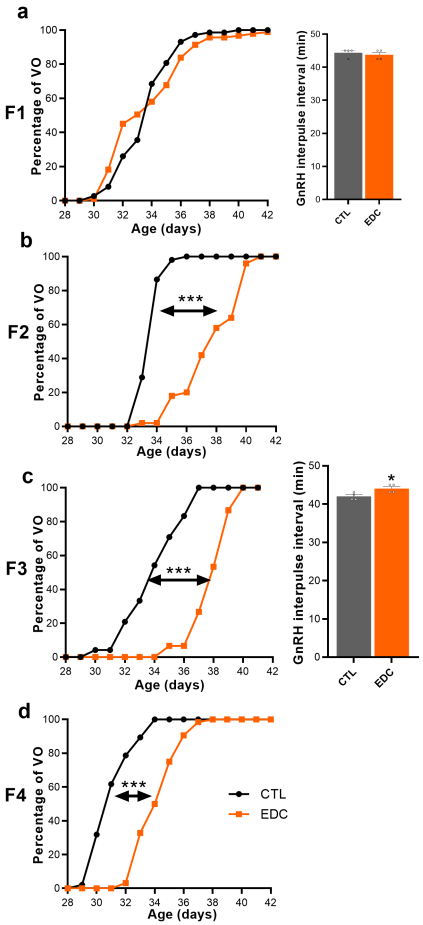


Figure 8.2. Pubertal timing (vaginal opening) and GnRH interpulse interval across generations (F1-F4 generation) after an EDC mixture exposure or vehicle (F0 generation). (a-d left) Cumulative percent day at vaginal opening (VO) of rats exposed to the mixture of EDCs *in utero* and through lactation (F1 generation, n=51-56/group), through germ-cell (F2 generation, n=50-52/group) or not being directly exposed (F3 generation, n=15-24/group; F4 generation, n=47-64/group). (a and c, right) GnRH interpulse interval measured in F1 and F3 generation females at P20 *ex vivo* through an hypothalamic explant incubation carrying out sequential sampling every 7.5 minutes for 4 hours in MEM followed by radioimmunoassay (n=4/group). Bars represent mean ∓ s.e.m. (*P < 0.05, ***P < 0.001 vs. CTL, Student's t-test. Statistical comparison of vaginal opening were carried out in mean average).

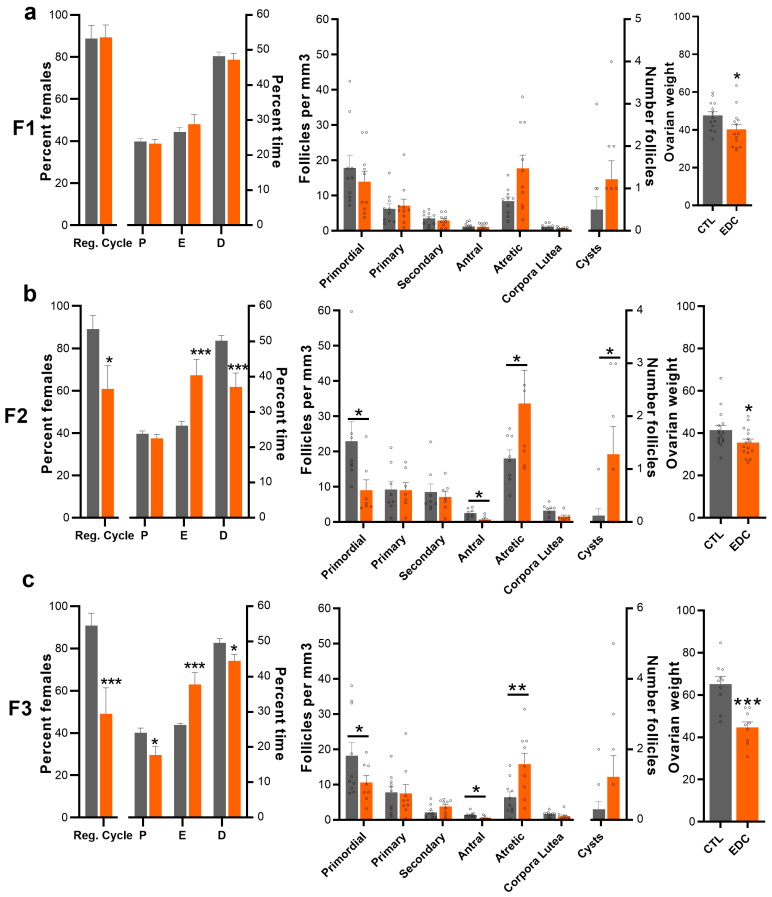


Figure 8.3. Estrous cycle, ovarian follicle development and ovarian weight across generation (F1-F3 generation) of female rats exposed to an EDC mixture or vehicle (F0 generation). (a-c left) Percentage of females having regular cycle and average time spent in different stages of the estrous cycle by rats exposed to the EDC mixture *in utero* (F1, n=20/group), through germ cell (F2, n=15/group) or ancestrally (F3, n=14-15/group). (a-c middle) Number of ovarian follicles per mm^3 throughout development, corporal lutea and cysts quantified at P60 in F1, F2 and F3 females (n=9-10/group). (a-c right) Ovarian weight measured at P60 in F1 (n=14/group), F2 (n=16/group) and F3 (n=9-10/group) females. Reg. cycle= regular cycle, P= proestrus, E= estrus, D= diestrus. Bars represent mean ∓s.e.m. (*P < 0.05, **P < 0.01, ***P < 0.001 vs. CTL, Student's t-test or two-way ANOVA)..

was detected at the *Pomc* promoter, suggesting that the potential role of EDC exposure in the epigenetic programing of the hypothalamus is locus specific (see supplementary appendix Fig.E.3).

EDC mixture disrupts the dopaminergic control of maternal behavior throughout generations

Phenotypic inheritance of EDC effects has been shown to be transmitted through epigenetic changes in germ-cells (Crews et al., 2012). Alternatively, maternal behavior is known to affect pubertal development (Cameron et al., 2008) and can be passed down throughout generations by inducing epigenetic changes at the somatic level. Because EDCs have been shown to affect maternal care (Boudalia et al., 2014), we aimed at exploring the effects of the EDC mixture on maternal care throughout generations. Direct exposure to the EDC mixture (F0 generation) did not alter maternal care (Fig.8.5.a). F1, F2 and F3-EDC dams displayed impaired maternal behavior characterized by lower levels of licking and grooming compared to the control dams (Fig.8.5.b-d). Additionally, females exposed *in utero* (F1 generation) or as germs cells (F2 generation) spent more time resting alone or being active outside the nest, respectively. No changes were observed in retrieval, nursing, nest building or in average in-nest activity throughout generations (see supplementary appendix Fig.E.4 to E.9).

In order to identify the hypothalamic targets potentially involved in the alterations of maternal care programming caused by EDCs, we searched the massive parallel RNA sequencing gene ontology analysis described before in MBH of F3-EDC females (see supplementary appendix Fig.E.2) focusing on those downregulated genes that belong to the enriched categories: social behavior, maternal behavior, grooming behavior and synaptic dopaminergic transmission (see supplementary appendix Fig.E.1). Additional gene ontology analysis of massive parallel RNA sequencing done in MBH of F1 females showed downregulated categories related to D2 dopamine receptor binding, glutamate binding and neurotransmitter uptake (see supplementary appendix Fig.E.8). qPCR validation of common target genes in F1 and F3 gene ontology analysis showed that critical dopaminergic signaling (*Th*, *Dnm1* and *Darpp32*), involved in maternal motivation, was significantly decreased in the hypothalamus of F1-EDC animals at P21 and/or 60 (Fig.8.6.a). On the other hand, the dopaminergic receptor 1 (*Drd1*) was found to

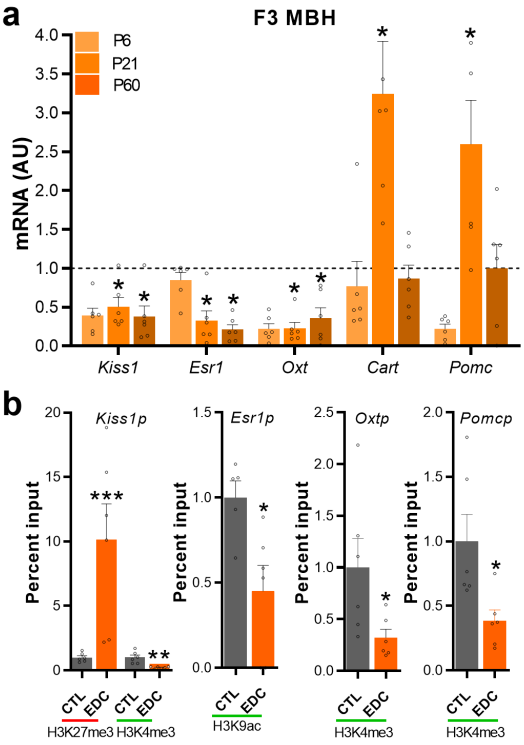


Figure 8.4. *Kiss1, Esr1, Oxt, Cart* and *Pomc* mRNA expression and promoter chromatin state in the female rat ancestrally (F3 generation) exposed to an EDC mixture or vehicle. (a) Expression of *Kiss1, Esr1, Oxt, Cart* and *Pomc* mRNA in the MBH of infant (P6), prepubertal (P21) and adult (P60) female rats as determined by qPCR (n=6/group). AU = arbitrary units. RNA expression data were normalized by dividing each individual value by the average of the control group at every time point. (b) Abundance of the TrxG-dependent activating marks H3K4me3 and H3K9ac and the PcG-dependent repressive mark H3K27me3 at the *Kiss1, Esr1, Oxt, Cart* and *Pomc* promoter in the prepubertal MBH of females ancestrally exposed to a mixture of EDCs (F3 generation), as measured by ChIP (n=6/group). Bars represent mean ∓ s.e.m. (*P < 0.05, **P < 0.01, ***P < 0.001 vs. CTL, Student's t-test)..

be upregulated, a possible sign of a compensatory mechanism induced by reduced synaptic dopamine (Fig.8.6.a). Moreover, genes associated with stress responsiveness (*Nr3c1* and *Crh*) were not altered (see supplementary appendix Fig.E.9.a). Overall, these data indicate that exposure to EDC decreased the expression of key genes involved in the dopaminergic control of maternal behavior.

Epigenetic analysis of the Th promoter (*Thp*) region identified no change in DNA methylation (Fig.8.6.b), but higher levels of the repressive histone H3K27me3 modification in the hypothalamus of F1-EDC females (Fig.8.6.c) without affecting H3K9me3, H3K4me3 or H3K9ac status. No alterations in histone modification landscape were detected in the 5'-regulatory regions of *Darpp32* or*Drd1* (see supplementary appendix Fig.E.3.b), suggesting that transcriptional alterations caused by EDCs do not involve epigenetic reprogramming in the promoter region of those genes and demonstrating that the epigenetic alterations are targeted to specific loci. To further characterize the loss in dopaminergic signaling, we quantified the TH-immunoreactivity in the substantia nigra (SN), the ventral tegmental area (VTA) and the median preoptic area (mPoA), part of the VTA-mPOA-NAc pathway involved in maternal motivation (Fang et al., 2018). Exposure to EDCs significantly decreased the number of TH immunopositive cells in the mPOA of F1 females at P21 (Fig.8.6.d-e), confirming the mRNA expression results. No differences were observed in the VTA or NAc. Because the F3 generation also showed diminished licking and grooming behavior as a result of the EDC mixture treatment of the F0 generation, we hypothesized that the dopaminergic system would also be affected. *Th* and *Drd1* mRNA expression were found to be significantly reduced in hypothalamus of F3-EDC females (Fig.8.6.f). *Th* downregulation was associated with increased DNA methylation at 3 specific CpGs in the *Th* promoter, as well as enhanced repressive H3K27me3 and H3K9me3 histone marks (Fig.8.6.g-h). No differences were found in the activating histone modifications H3K4me3 and H3K9ac. These results indicate that the transgenerational reduction of licking and grooming behavior is also linked to diminished hypothalamic dopaminergic signaling by increased action of the Polycomb group (PcG) of epigenetic repressors, and maybe other histone methyl transferases on the *Th* promoter.

So far, our data show a direct effect of the EDC mixture on the prenatal

development of the F1 generation's dopaminergic system throughout diminished dopamine signaling and reduced maternal behavior. The perpetuation of this phenotypic manifestation throughout generations, even in the absence of EDC exposure, is also associated to hypothalamic dopaminergic loss, but through epigenetic reprogramming mediated by the PcG and increased DNA methylation at specific CpGs in the *Th* promotor.

Sexual maturation is altered through germ-cell transmission

Our data identified a multigenerational transmission of maternal care starting with the F1 all the way down to the F3 generation. Sexual maturation was found to be delayed in F2 to F4 generations and associated with epigenetic reprogramming of the hypothalamus identified in the F3 generation. As no differences in sexual maturation was found in *in utero* exposed females (F1 generation), we hypothesized that the delay in sexual maturation in F2 and consecutive generations could be explained by hypothalamic reprogramming caused by variations in maternal care. To determine whether delayed puberty is caused by germ-cell or experience-based inheritance, a cross-fostering paradigm was carried out. Control F2 pups raised by CTL (CC) or EDC (EC) F1 dams showed normal vaginal opening and estrous cyclicity. To the contrary, germ-cell EDC exposed pups (F2 generation) raised by an EDC (EE) or CTL (CE) dams, indistinctively showed a delay in vaginal opening (Fig.8.7.a) and disrupted estrous cycles, characterized by increased time spent in estrus and decreased time spent in diestrus (Fig.8.7.b). F2 germ-cell exposed pups showed a downregulation in the hypothalamic expression of *Kiss1*, *Esr1* and *Oxt* at P21, independently of being raised by an EDC (EE) or control (CE) dam and these changes persisted through the next generation (Fig.8.7.c-f). Downregulation of *Kiss1*, *Esr1* and *Oxt* was consistently associated with abundance of histone marks, showing a repressive state (Fig.8.7.g-I, see supplementary appendix Fig.E.10). These results show that cross-fostering could not restore normal pubertal timing in germ-cell EDC exposed animals, nor did EDC exposed dams cause delayed sexual maturation of unexposed pups. Our results demonstrate that delayed sexual maturation is caused by a germ-cell inheritance of EDC mixture effects.

Here we also show that in F1 females, the dopaminergic system abnormalities

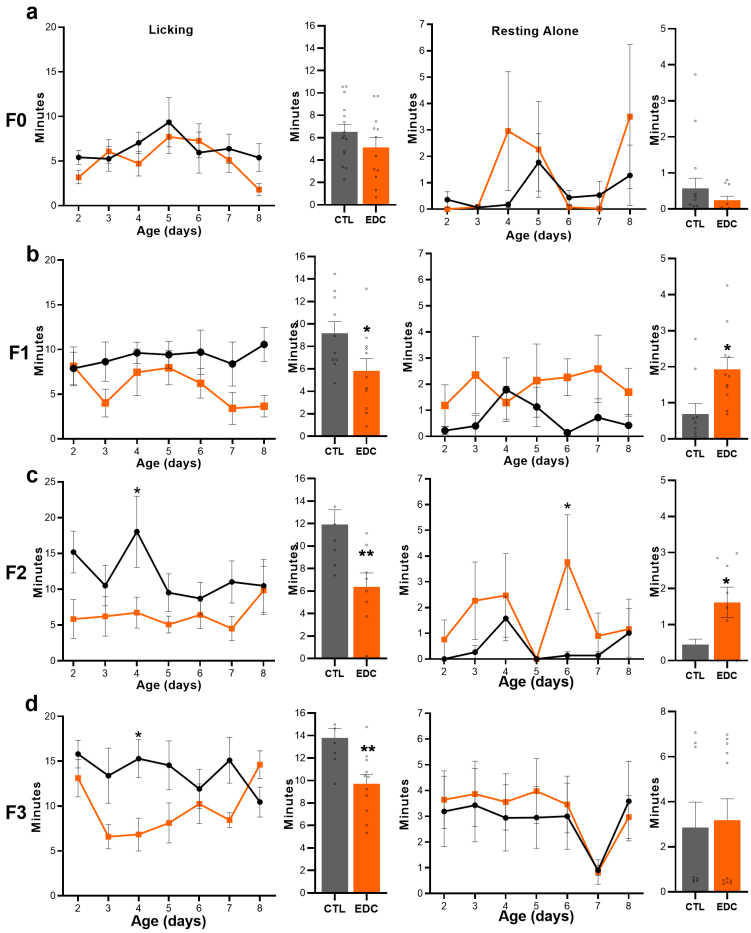


Figure 8.5. Maternal behavior displayed by female rats exposed to a mixture of EDCs throughout 4 generations. (a-d left) Time spent by dams displaying licking and grooming behavior toward pups after direct (F0 generation, n=15/group), *in utero* and through lactation (F1 generation, n=10-11/group), germ-cell (F2 generation, n=11/group) or ancestral (F3 generation, n=11/group) exposure to an EDC mixture or vehicle from P2 to 8. Bar graph shows pooled time licking and grooming from P2-8. (a-d right) Time spent by dams resting alone outside the nest not being involved in maternal care throughout generations (F0-F3 generation). Bar graph shows pooled time spent resting alone from P2-8. Plotted lines represent average of time ∓ s.e.m. (*P < 0.05 vs. CTL, two-way ANOVA followed by Sidak's multiple comparisons test).

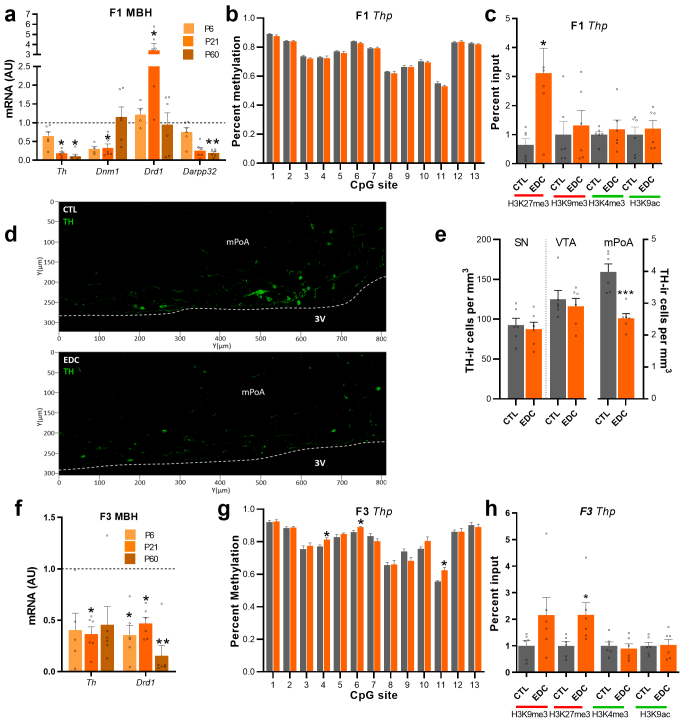


Figure 8.6. Dopaminergic signaling proteins, mRNA expression and chromatin state in the female rat *in utero* (F1 generation) and ancestrally (F3 generation) exposed to an EDC mixture or vehicle. (a) Expression of *Th, Dnm1, Drd1* and *Darpp32* mRNA in the MBH of infant (P6), prepubertal (P21) and adult (P60) female rats *in utero* and lactationally (F1 generation) exposed to an EDC mixture as determined by qPCR (n=6/group). RNA expression data were normalized by dividing each individual value by the average of the control group at every time point. (b) Methylation state at 13 CpG sites of the *Th* gene promoter from MBH explants of F1 females at P2 (n=6/group). (c) Abundance of the TrxG-dependent activating marks H3K4me3 and H3K9ac and the PcG-dependent repressive mark H3K27me3 and H3K9me3 at the *Th* promoter in the prepubertal MBH of females EDC and control from the F1 generation, as measured by ChIP (n=6/group). (d) Abundance of TH-ir cells (green color) within the mPoA of prepubertal female rats. 3V, third ventricle. (e) Quantification of TH immunoreactivity in the SN, VTA and mPoA of females from the F1 generation. Bars represent mean of cells per mm^3 ∓ s.e.m; (f) Expression of *Th* and *Drd1* mRNA in the MBH of infant (P6), prepubertal (P21) and adult (P60) female rats ancestrally (F3 generation) exposed to EDC mixture (n=6/group). (g) Methylation state of at 13 CpG sites of the *Th* gene promoter from MBH explants of F3 females at P2 (n=6/group) (h) Abundance of H3K4me3,H3K9ac, H3K27me3 and H3K9me3 histone posttranslational modifications at the *Th* promoter in the prepubertal MBH of females EDC and control from the F3 generation, as measured by ChIP (n=6/group). AU = arbitrary units. Bars represent mean ∓ s.e.m. (*P < 0.05, **P < 0.01, ***P < 0.001 vs. CTL, Student's t-test).

are associated with alterations in maternal care. Our cross-fostering paradigm reveal that *Th* mRNA levels are downregulated in F2 and F3 generations when raised by an EDC-exposed mother, independently of their ancestral as germ cells (EE or EC) exposure, suggesting that the alterations in the dopaminergic system are transmitted through maternal care (Fig.8.7.f). *Th* downregulation was associated with enhanced repressive H3K27me3 histone marks as found in the previous experiment (Fig.8.7.j, see supplementary appendix Fig.E.10).

Overall, our results demonstrate that direct EDC mixture action on F1 females during development diminishes hypothalamic dopaminergic signaling impacting licking and grooming behavior. This behavior is "learned" as it seems to be transmitted down to the next generations. On the other hand, the delayed sexual maturation is transmitted throughout generations and cannot be corrected when pups are cross-fostered with controls dams. These results suggest a germline transmission of the reproductive phenotype.

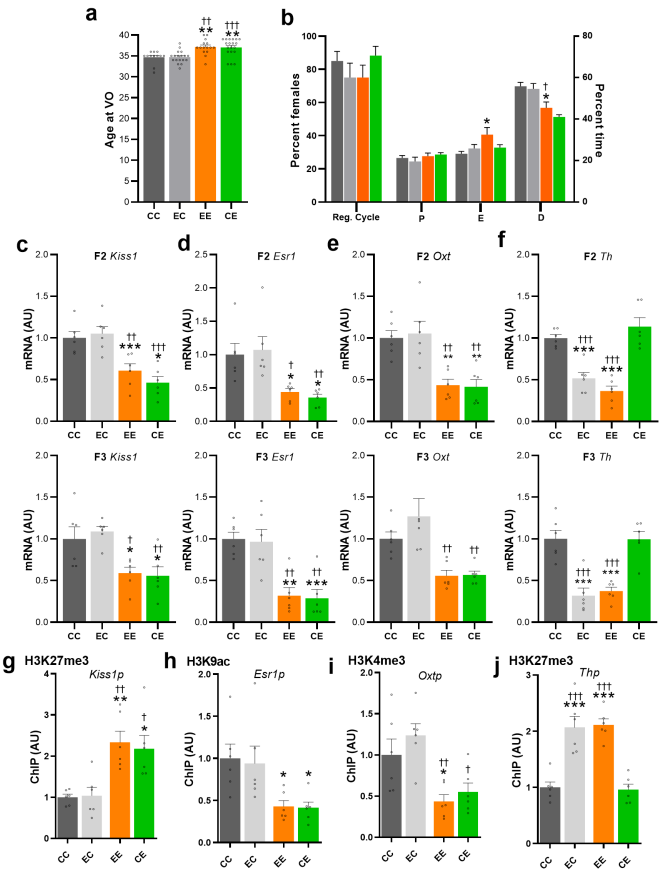


Figure 8.7. F2 cross-fostered offspring sexual maturation and mRNA expression data. (a) Average age at vaginal opening of cross-fostered germ-cell EDC exposed pups or control raised by either *in utero* EDC exposed dams or control (n=12-19/group) (b) Percentage of cross-fostered females having regular cycle and time spent in different stages of the estrous cycle (n=10/group) (c-f) Expression of *Kiss1 Esr1, Oxt* and *Th* mRNA in the MBH of F2 and F3 generation crossfostered pups at P21, as determined by qPCR (n=6/group). (g-j) Abundance of the TrxG-dependent activating marks H3K4me3, H3K9ac or the PcG-dependent repressive mark H3K27me3 at the *Kiss1, Esr1, Oxt* and *Th* promoter in the prepubertal MBH of females crossfostered from the F3 generation, as measured by ChIP (n=6/group). Reg. cycle= regular cycle, P= proestrus, E= estrus, D= diestrus. CC= control pup raised by control dam; EC: control pup raised by *in utero* EDC exposed dam; EE= germ-cell EDC exposed pup raised raised by *in utero* EDC exposed dam; CE= germ-cell EDC exposed pup raised by control dam. Bars represent mean ∓ s.e.m. (*P < 0.05, **P < 0.01, ***P < 0.001 vs. CC; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. CE, one-way ANOVA).

8.4 Discussion

The potential contribution of developmental exposure to a low dose EDC mixture in the neuroendocrine regulation of female reproductive and maternal behaviors, to the best of our knowledge, has never been addressed. In the present report, we provide evidence that EDC exposure induces multi- and trans-generational alterations of maternal care and sexual maturation, respectively, throughout epigenetic reprogramming of the hypothalamus. Our results identify a delay in pubertal development that appears apparent starting at the F2 generation, in females exposed to the EDC mixture through the germline. The reproductive phenotype is found up to the F4 generation and is determined as a delay in the day of vaginal opening and increase in the GnRH interpulse interval. These animals showed disrupted estrous cyclicity, with increased time in estrus and decreased time in proestrus and diestrus, a sign of diminished ovulatory cycle efficiency. Moreover, EDC exposed animals showed a significant reduction in antral and enhanced atretic follicles, a clear sign of subfertility. Surprisingly, no reproductive effects were detected in the F1generation, the one directly exposed to the EDC mixture during embryonic and lactational development, which suggests that the reproductive phenotype appears as a consequence of germline exposure.

The activation of the GnRH network during the juvenile period involves the transcriptional activation of KNDy neurons, those that co-express Kisspeptin, Neurokinin-b and Dynorphin. We have recently deciphered that female sexual maturation is controlled by the dual coordinated action of the Polycomb (PcG) and Trithorax (TrxG) group of chromatin remodelers, leading to a concomitant epigenetic and transcriptional switch from repression to activation of the *Kiss1* locus in KNDy neurons of the ARC (Toro et al., 2018; Lomniczi et al., 2013b). Moreover, we recently described that metabolic cues target KNDy neurons through epigenetic modifications affecting pubertal timing (Vazquez et al., 2018). Specifically, undernutrition-induced delayed pubertal onset was found to be related to a developmental increase in the repressive histone deacetylase Sirtuin 1 (SIRT1) responsible for decreased H3K9/16ac at the *Kiss1* gene promoter, as well as an increase in H3K27me3, a repressive histone modification related to the activity of the PcG of epigenetic repressors. Since developmental exposure to the plasti-

cizer BPA can disrupt kisspeptin neurons in adults (Losa et al., 2011; Losa-Ward et al., 2012), we hypothesized that one putative target of EDC mixture induced disruption of puberty could be the KNDy neuron. RNAseq analysis of the hypothalamus of F3 females ancestrally exposed to the EDC mixture demonstrated that the reproductive effects where associated with decreased *Kiss1*, *Oxt* and *Esr1* expression, three main players in the control of the GnRH pulse generator and the correct timing of puberty and ovulation (Seminara et al., 2003; Clarkson and Herbison, 2011; Parent et al., 2008; Yeo and Herbison, 2014; Wintermantel et al., 2006). Here we found that F3 descendants of EDC treated animals show a disbalanced histone configuration at the *Kiss1* promoter with increased repressive H3K27me3 and reduced H3K4me3, a histone modification found at promoter regions of activated genes, suggesting a central role of the PcG/TrxG balance in the EDC induced delay of puberty. Moreover, although we found that the hypothalamic expression of *Pomc* and *Cart*, two postulated metabolic activators of the GnRH system (Roa and Herbison, 2012; Parent et al., 2000), is transiently increased, it did not overcome the effects of the *Kiss1*, *Esr1* and *Oxt* loss on pubertal delay. The loss in *Esr1* expression was associated with the diminished presence of H3K9ac at its promoter region, suggesting a role of the sirtuin deacetylases as putative regulators of EDC mediated reproductive effects. Our results suggest that EDC mixture exposure affects the epigenetic programming on the hypothalamus throughout generations delaying sexual maturation and altering estrous cyclicity and folliculogenesis. A similar transgenerational phenotype was observed after developmental exposure to pesticides, jet fuel, DEHP and TCDD, displaying altered puberty, lower primordial follicles and accelerated follicle recruitment (Pocar et al., 2017; Manikkam et al., 2012a; Nilsson et al., 2012; Manikkam et al., 2012b). Furthermore, there are striking similarities between the epigenetic changes involved in the EDC induced reprogramming of the hypothalamus and those induced by undernutrition. In both cases we identified a predominant role of the PcG and sirtuins in repressing gene expression by increasing the repressive role of H3K27me3 and decreasing the effects of H3K9ac at the promoter region of puberty activating. This observation is in line with several reports suggesting that EDC function as metabolic disruptors by sharing common intracellular pathways (Heindel et al., 2017).

It is known that alterations in maternal care are transmitted through generations affecting the hypothalamic response to stress (Schmauss, Lee-McDermott, and Medina, 2014) and also impacting reproductive development by modulating *Esr1* expression (Peña, Neugut, and Champagne, 2013). Our ancestrally EDC mixture exposed (F2-F3) animals showed delayed puberty of central origin and diminished pup licking behavior, without affecting any other maternal behavior. Surprisingly, the F1 generation, that had normal pubertal development and ovarian phenotype, also showed diminished pup licking behavior, suggesting that direct EDC mixture exposure during intrauterine development directly affects the neuronal network involved in parental behavior. Gene ontology analysis of hypothalamic RNAseq demonstrated that the dopaminergic system is one of the main targets of the EDC mixture in the F1 generation. In particular, EDC exposed F1 females showed a significant loss in *Th* expression accompanied with increased repressive H3K27me3, a main target of PcG action. Moreover, this effect is localized to the mPoA, since there was no TH staining loss in either the SN or the VTA, demonstrating that the action of the EDC mixture is region specific and directed to epigenetically reprogram the dopaminergic system. On the other hand, females of the F3 generation also showed diminished dopaminergic signaling in the hypothalamus, but with further penetrance of the epigenetic phenotype. In this case, the *Th* promoter not only showed increased repressive H3K27me3 but also H3k9me3, as well as increased CpG methylation at 3 independent sites throughout the *Th* promoter. This shows that the epigenetic reprogramming of the *Th* locus of the F3 generation differs of that of the F1, since the F3 generation was not directly exposed to the EDC mixture but it was exposed to a dysfunction in maternal care, raising the possibility of direct multigenerational transmission of the behavioral phenotype. Such alteration is multigenerationally transmitted through epigenetic alteration of the dopamine system.

In the current study, we have found a transcriptional and epigenetic alteration of stress response genes in F3 females raised under diminished maternal care. F1-EDC females did not show differences in hypothalamic *Nr3c1* or *Crh* expression, while receiving normal maternal care from their F0 mothers. This confirms previous studies showing that maternal care induces increased responses of the HPA axis, decreasing *Nr3c1* transcriptional levels via epigenetic alterations throughout

generations (Weaver et al., 2004; Liu et al., 1997).

We also identified that the reproductive phenotype is transmitted through the germ line and is not explained by impaired maternal behavior, since the cross-breeding of the F2 generation with non-exposed mothers did not normalize pubertal timing nor the downregulation of hypothalamic *Kiss1*, *Esr1* and *Oxt* expression in the F2 or F3 generations. These effects are not general since the cross-fostered EDC exposed animals raised by control females showed a normalization of the hypothalamic dopaminergic network by increased *Th* expression, indicating that the reduction in maternal behavior is in fact learned and multigenerationally transmitted from the F1 generation. The transgenerational disruption of sexual maturation phenotype could be caused by germ cell alterations of epigenetic developmental programs induced by EDC treatment. This EDC-induced epigenetic reprogramming needs to be resistant to erasure and to be transmitted across multiple generations (Jirtle and Skinner, 2007). Thereafter, EDC-induced germ cell alterations flow from germline to soma (Lim and Brunet, 2013), affecting in this case the organization of the GnRH network. It is possible that environmental factors alter the germline epigenome directly or indirectly through soma-to-germline transmission. For instance, it has been recently reported that small RNA molecules are transferred from somatic cells to germ cells, and that EDC treatment affects ncRNAs in germ cells (Cossetti et al., 2014; Chen, Yan, and Duan, 2016; Brieno-Enriquez et al., 2015). Further studies should address these issues and determine the developmental window at which the germ cells epigenome is more sensitive to environmental alterations.

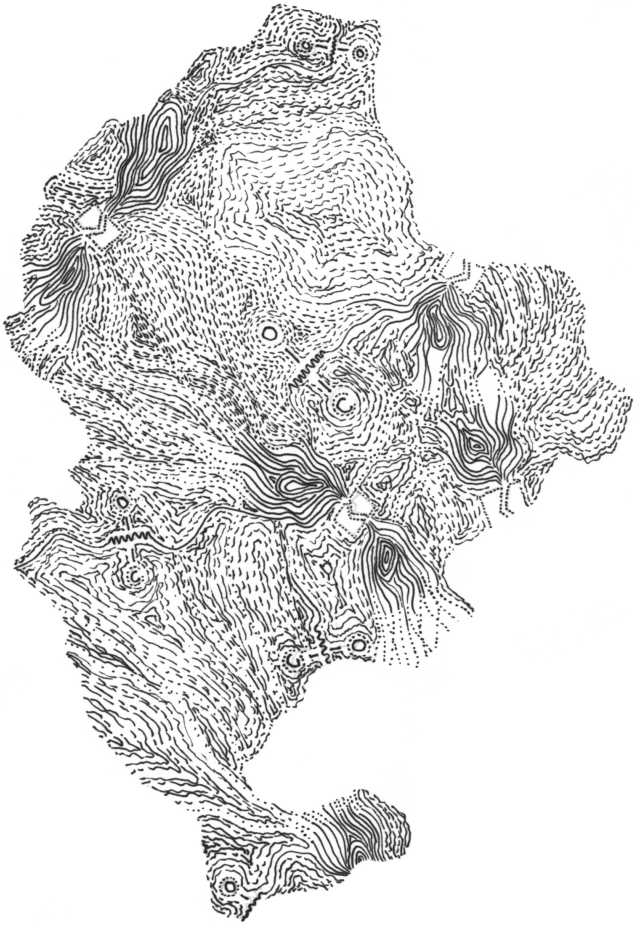
In current human pregnancy conditions, virtually any woman and her fetus are exposed to a low-dose mixture of at least 100 EDCs (Gore et al., 2015). Small-scale studies with women visiting fertility clinics show that human follicular fluid samples contain a wide array of chemicals with endocrine disrupting activity, such as DDT, phthalates, bisphenol A and perfluorinated compounds, indicating direct exposure of maturing oocytes and their surrounding steroid-producing cells (Petro et al., 2014; Petro et al., 2012; Craig, Wang, and Flaws, 2011). Most studies are not designed to investigate environmentally relevant combinations. However, such studies are crucial from a regulatory point of view because current risk assessment is based on the effects of individual chemicals. In order to address the

issue of complex mixture, we have selected chemicals for which rudimentary information about their endocrine disrupting effects *in vivo* and data about human exposures were available to guide the choice of doses (Christiansen et al., 2012). Developmental exposure to this mixture at high doses has been previously shown to alter sexual differentiation in males and disrupts estrous cyclicity in females when exposed *in utero* (Johansson et al., 2016; Isling et al., 2014). However, this study is the first one to our knowledge to report trans- and multi-generational effects of a low dose mixture on the hypothalamic control of puberty and maternal behavior.

8.5 Conclusions

Altogether, the present results demonstrate that developmental exposure to a human relevant dose of an EDC mixture alters the hypothalamic epigenetic programming of reproduction through increased action of the PcG and possibly sirtuins on key puberty activating genes and that these effects are transgenerationally transmitted. In addition, direct exposure to the EDC mixture downregulates the hypothalamic dopaminergic network by increased action of the PcG and other methyltransferases, affecting maternal behavior in a multigenerational and reversible manner.

GENERAL DISCUSSION



9 General discussion

Contents

This PhD thesis aimed at characterizing the impact of EDCs -as a single compound or in mixture- on the hypothalamic control of female sexual maturation and maternal care. We used low environmental doses for each compound in order to mimic human exposure. We (1) compared BPA effects on the neuroendocrine control of female reproduction different different windows of exposure and (2) investigated the effects of a developmental exposure to a mixture of EDCs on maternal behavior and sexual maturation throughout generations. In this discussion, we will start with a short overall assessment of the data collected in this PhD thesis, followed by an explanation of the main limitations of our model and the future perspectives of the project.

9.1 Overall assessment

We showed that exposure to BPA or an EDC mixture alters the neuroendocrine system at different levels. The window of exposure appears to be determinant in both projects. In the first experimental study, we showed that BPA exposure during early postnatal development or adulthood could impact the hypothalamic control of female reproduction. However, impacts differ with the window of exposure. Early postnatal exposure to BPA disrupts the organization of the hypothalamus. Such alteration is consistent with the developmental origin of health and disease hypothesis (DOHaD or barker hypothesis) and indicates that environmental factors interfere with hypothalamic organizational events critical for the normal reproduction. The delay in puberty and alterations in estrous cyclicity documented in previous (Franssen et al., 2016) and current reports (Lopez-Rodriguez et al., 2019) from our laboratory are likely to be explained by such organisational disruption. Such long term consequences of BPA on adult female reproductive functions after early developmental exposure have been previously reported by others (Fernandez et al., 2009; Monje et al., 2010; Nah, Park, and Gye, 2011; Wang, Hafner, and Flaws, 2014). However, no study so far had reported effects at such low and environmentally relevant doses.

We compared the results obtained after early developmental exposure with adult exposure to BPA and showed that 24 hours after adult exposure, the LH preovulatory surge, estrous cyclicity and folliculogenesis were disrupted. Additionally, a modest alteration of GnRH secretion was found. These effects were transient and did not persist beyond BPA exposure, with females recovering a few weeks after the end of exposure. These results are consistent with activational mechanisms of action. Here, BPA appears to target a system already in place and disrupt its homeostasis. It is important to note that short term exposure to BPA -and more likely to any EDC- early during development or at adulthood does not represent reality as humans are continuously exposed throughout their lifespan. With that caveat, these results reinforce the notion of neuroendocrine disruption of reproduction caused by EDCs and demonstrate the importance of limiting BPA exposure during early development but also later in life.

In our second study, we exposed female rats (F0 generation) to a mixture of 13

EDCs from 2 weeks before gestation until the end of lactation and we examined the neuroendocrine and behavioral consequences throughout four generations (F1-F4). This window of exposure -together with low doses- were selected to mimic real world situations. Interestingly, EDC effects were only observed from the second generation (F2). *In utero* exposure (F1 generation) to the EDC mixture did not appear to affect the neuroendocrine control of maternal behavior, sexual maturation or reproduction. However, direct female germline exposure was able to disrupt the hypothalamic control of puberty and reproduction, and this effect was transmitted across generations. These data have important implications as it suggests that the impact of EDCs could only start to be appreciated two generations after the exposure, and thus, be a consequence of germline exposure.

As we have seen in the introduction of this dissertation, the female germline develops from embryonic day 7 in the yolk sac and maintains meiosis throughout migration and selection until they reach the gonadal ridge. Epigenetic reprogramming of these cells happens around embryonic day 13 in mice (Smith et al., 2012). The experimental study carried out in this dissertation suggests that EDC exposure may directly target germline development of F1 females, affecting their development or reprogramming, ultimately having an impact on the pool of germ cells that will lead to the F2 generation. The implementation of a crossfostering paradigm in our model further supports this hypothesis. This crossfostering study allows us to exclude maternal care as a potential source of non-genomic transmission of the reproductive axis.

The mechanisms by which EDCs may affect the germline are largely unknown, and have been studied mostly in males (Guerrero-Bosagna et al., 2013; Skinner et al., 2013; Manikkam et al., 2012a). While males are vulnerable to environmental factors during all their reproductive lifespan, the female germline is vulnerable during a short period of time during embryogenesis and early postnatal life, from the initiation of the female germline to their differentiation into oocytes, when they remain in a state of meiotic arrest. During this period, environmental factors such as EDCs could directly or indirectly interact with the germline leading to transgenerational alterations of inherited information. While difficult to study, a few hypotheses have attempted to explain the transmission of a phenotype through the germline: (1) by modifying the number of loci that are

protected from epigenetic reprogramming. Some loci, such as imprinted genes, contain retrotransposons or loci in close proximity to intracisternal A-particles (IAPs). These are protected or resistant to CpG methylation erasure (Hackett et al., 2013). So far, the mechanisms leading to specific loci to escape reprogramming are not clear. However, regions of the genome containing high density of epigenetic information (i.e. CpG regions or binding sites for chromatin remodelers) may play an important role in preventing specific loci to be reprogrammed (Grossniklaus et al., 2013; Osborne et al., 2014). (2) By modifying the expression of non-coding RNAs such as PIWI-interacting RNAs, whose expression is inherited transgenerationally (Ashe et al., 2012; Brennecke et al., 2008). Modifications in RNA may be responsible for the transmission of the agouti viable yellow allele (A^{vy}) in mice (Daxinger and Whitelaw, 2012). The A^{vy} allele is a results of a IAP insertion upstream of the agouti locus. The transcriptional control of the agouti gene depends on promoter elements in the IAP retrotransposon (Duhl et al., 1994). The degree of methylation at the IAP promoter of the dominant A^{vy} allele determine coat color and is associated with obesity, type II diabetes and predisposition to cancer (Morgan et al., 1999a). Furthermore, this phenotype is transgenerationally transmitted through the maternal germline. The presence of sncRNA could explain the silencing of loci containing retrotransposons such as the A^{vy} allele, inducing its transgenerational inheritance (Osborne et al., 2014; Daxinger and Whitelaw, 2012). (3) By modifying expression of mitochondrial DNA. Mitochondrial DNA has very few CpG sites and the role of these CpGs in the normal functioning of the mitochondria remains unknown (Shock et al., 2011). However, mitochondrial dysfunction affects the methylation state of nuclear genes (Minocherhomji, Tollefsbol, and Singh, 2012). Thus, alterations in mitochondrial function can be a mechanism of transgenerational inheritance through the female germline. (4) By modifying soma-to-germline interactions. Placental environment can be modulated by hormonal signals and environmental factors. EDCs could affect somatic populations in the placental tissue and have consequences on germline development (Fowden and Forhead, 2009).

Epigenetic information transmitted through germline explain the predisposition to develop some disease across generations and have major consequences for our society as well as for species evolution. Our results clearly show that EDC

mixture exposure during vulnerable window of development has consequences far beyond the lifespan of exposed individuals. Whether the exposure to BPA in the first experimental study can affect the germline and have transgenerational consequences is not known. Thus, further studies will have to address this question. At present, only one study has reported transgenerational consequences of BPA exposure in the neuroendocrine system. Developmental exposure to BPA was found to induce transgenerational alterations in the expression of ERα in the AVPV of the hypothalamus (Goldsby, Wolstenholme, and Rissman, 2017).

9.2 Limitations of the project

Endocrine disruption studies imply a series of limitations resulting from methodological choices. We list here some of these limitations.

Selection of window of exposure. As stated in the previous section, human exposure is not limited to a specific period but rather extends throughout the whole lifespan. In the two experimental studies carried out during this thesis, we aimed at determining the long-term and transgenerational consequences of EDCs after a developmental exposure and the direct effects of EDCs after adult exposure. This approach requires to limit exposure to specific windows, in order to differentiate between the organisational, activational and transgenerational effects of EDCs. This aim was achieved in the first experimental study by choosing BPA, a non persistent compound with a short half life. All measured outcomes (i.e. vaginal opening ,estrous cycle) were measured after the end of exposure. Strict experimental conditions allow us to consider that BPA was not present anymore when outcomes were measured. In the second experimental study, we privileged compounds representing human exposure, independently of their persistence. For instance, p'p'-DDE, is the main metabolite of the insecticide DDT. It is a persistent compound (Jackson et al., 2017) and it has been associated with metabolic (Arrebola et al., 2013) and reproductive (Cohn et al., 2003) disorders in humans. p'p'-DDE half-life is very long, which explains a persistence long after the period of exposure.

Transgenerational inheritance. Detection of true transgenerational inheritance not only requires to study at least the third generation but also to control for non-genomic mechanisms of transmission. In our study, we have controlled for

maternal care by carrying out a cross-fostering paradigm as it is known that similar experiences can cause similar epigenetic marks between parents and offsprings (Weaver et al., 2004). Such effect is not heritable but transmitted by experience to the next generation. Furthermore, intrauterine environment is suspected to play a role in the transmission of epigenetic information (Buss et al., 2017). The embryonic and fetal life is particularly sensitive to environmental stressors. For instance, EDCs, stress, alcohol consumption or other factors affect the intrauterine environment and could lead to multigenerational alterations. While mechanisms are still incompletely understood, transgenerational studies may need to include embryo transfer to their experimental design to control for potential effect of the intrauterine environment.

Mechanisms of EDCs. BPA estrogenic action has been largely described but the compound is also known to have antiandrogenic activity and to interact with other nuclear receptors such as AhR (Acconcia, Pallottini, and Marino, 2015). We have found that alterations in the LH preovulatory surge explaining the disruption of estrous cyclicity after adult BPA exposure may be caused by deregulation of circadian clock genes. Interestingly, it is known that clock gene oscillations are independent of oestrogen levels (Smarr, Gile, and Iglesia, 2013), suggesting that BPA may interact with these genes through alternative pathways. Thus, alteration of circadian clock genes after adult exposure to BPA would need to be studied further with the measurement of per1 and *bmal1* oscillations at different timepoints.

Global agencies such the European Commission have been urging scientist to investigate the transgenerational impact of EDC mixtures (Demeneix and SLAMA, 2019), as we have done in the second experimental study. However, the use of a mixture of compounds and the transgenerational model render the identification of specific mechanism of action impossible. Globally, the compounds included in the EDC mixture are known to be antiandrogenic (Christiansen et al., 2009). Each compound would need to be studied separately to determine the synergistic or additive nature of effects or whether a single compound could explain the reproductive phenotype.

Control of contamination factors. EDC studies require an extensive control of contamination factors. Given the ubiquitous presence of some EDCs in ma-

terials commonly used in a laboratory environment, precautionary measures are required. In both experimental studies we used EDC free cages, glass water bottles and low phytoestrogen chow. Additionally, *ex vivo* incubation of hypothalamic explants was done in glass tubes. While these factors were taken into account, we could not control for the presence of EDCs in running water.

Route of exposure. In the first experimental study, we selected subcutaneous injection as route of exposure for BPA. While the main route of exposure of BPA is through oral ingestion, this decision was taken to achieve greater precision regarding the administered dose, as it was required for the low dose of 25 ng/kg/d. Furthermore, oral exposure through food or water was not possible as exposure was done early in development when pups are still dependent of the mother. Gavage exposure was not chosen as it is stressful and alters estrogen receptors gene expression in the hypothalamus (Cao et al., 2012). In the second experimental study, EDC mixture was administered through food by adding the mixture to a waffle. While this method represent a non-invasive and non-stressful method of exposure it has some limitations. Human exposure to some of the chemicals in the mixture does not happen orally as this is the case for UV filters and parabens. As a consequence, exposure to these EDCs is likely reduced compared to natural conditions as all chemicals undergo the first pass effect.

Inter-species differences in sexual maturation. Our studies used rats as experimental model to measure pubertal timing. The rat has been extensively used for this purpose because of the similarities to humans in ovarian maturation, the neuroendocrine control of GnRH and the ability to measure GnRH secretion *ex vivo*. However, significant differences include the latency between birth and pubertal onset. While the average interval between birth and puberty represents 16.3% of the lifespan in humans, it represents 4.8% in rats (Parent et al., 2005). Additionally, inter-individual variance in pubertal timing is considerably more important in humans (4.8 years in humans) compared to rats (4-5 days) (Parent et al., 2005). Additionally, kisspeptin populations in human appear to be slightly different (Lehman, Hileman, and Goodman, 2013) and their epigenetic regulation remains unknown.

Guidelines of regulatory agencies. EDCs produce non-monotonic dose responses and environmental agencies so far have failed to take this phenomenon

into account. Doses used in our studies are hundred or thousands of times lower than the LOAEL and represent human exposure. However, a single dose of each compound was tested which does not allow for identification of potential non-monotonic dose responses.

Replicability crisis of science. In recent years, the scientific community has started a discussion around the issue of reproducibility. Many important findings in biomedical sciences -as in many other fields- could not be replicated (Ioannidis, 2005; Mullane, Curtis, and Williams, 2018). This issue is explained by common methodological deficiencies and underlines the importance of correctly reporting data analysis in scientific journals. In both of our experimental studies, most of the results (i.e. vaginal opening, estrous cycle, maternal behavior, developmental weight) have been replicated at least three times. While we consider sample size and power calculations, the final choice of number of animals assigned to each condition depends on practical limitations. Data was always analyzed taking into account the most suitable statistical test (depending on normality and homoscedasticity), followed by the calculation of effects sizes.

9.3 Future directions

Based on the results of our experimental studies, we could suggest the following perspectives.

In the first experimental study, we have found an alteration of circadian clock genes that could explain the disruption of the preovulatory LH surge after adult BPA exposure. Interestingly, BPA caused a systematic delay of the preovulatory LH surge during the proestrus afternoon. These results suggest alterations in the oscillation of the circadian clock controlling reproduction. However, to further explore this possibility, measurements of clock gene expression at different time points should be carried out.

In the second experimental study, to further demonstrate that the reproductive effects of the mixture of EDCs are caused through transmission of epigenetic information through the female germline, we could carry out a series of experiments such as: (1) limiting the timing of exposure to critical window for epigenetic reprogramming of the germline in females. (2) studying the presence of

transposable elements in specific cell populations of the hypothalamus such as the kisspeptin neurons. The presence and accumulation of new insertions in somatic hypothalamic cells of EDC exposed females compared to control are a reflection of germline alterations. As exposure to the EDC mixture lead to irregular estrous cycles and abnormal folliculogenesis, long term fertility should be explored.

EDC mixture exposure transgenerationally downregulated the expression of genes involved in the hypothalamic control of metabolism, such as *Cart* and *Pomc*. Because we found a downregulation of *Kiss1*, KNDy neurons would be an interesting population to study as they are known to be key players in the metabolic control of reproduction. For this reason, a future project could aim at (1) identifying and characterizing the transgenerational impact of an exposure to the EDC mixture on the hypothalamic control of metabolic functions in adults. For that, the developmental body weight, insulin and glucose tolerance and adiposity index could be measured. Furthermore, hypothalamic response to leptin and quantification of KNDy neurons could be performed. This approach can be followed by the identification of molecular hypothalamic metabolic targets and their chromatin state using RNAseq and ATACseq.

While we focused our attention on the central mechanisms of EDCs, we cannot exclude a peripheral effect. In both experimental studies we have identified alterations in ovarian follicle development, follicle atresia or the presence of cysts after exposure to BPA or an EDC mixture. Some of these results evoke a polycystic ovarian like-syndrome that could be further characterized by measuring testosterone levels after BPA exposure and to study steroidogenesis by theca and granulosa cells.

We observed multigenerational alteration of maternal care after exposure to an EDC mixture. Our data suggests that the EDC mixture exposure alters the dopaminergic pathway involved in maternal motivation in the F1 generation expose *in utero*. Future studies could confirm this hypothesis by confirming whether maternal motivation is specifically altered throughout consecutive generations. For that, adult primiparous females from each generation could be placed in a cage with pups in order to measure retrieval time and aggression. Time to retrieve is a well known marker of maternal motivation. Furthermore, future studies could address the involvement of hypothalamic dopaminergic neurons in alterations of

maternal care observed during this thesis. For that, a lentiviral vector expressing *Th* expression could be injected stereoxically in the hypothalamus of females exposed to the EDC mixture in order to test the possibility of reversal of the maternal phenotype.

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Appendix A

Review One

Cellular and molecular aspects of developmental neuroendocrine disruption of the GnRH network

nature REVIEWS

Nature Reviews Endocrinology (Accepted manuscript)

Cellular and molecular aspects of developmental neuroendocrine disruption of the GnRH network

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KEY POINTS

- Endocrine disrupting chemicals interfere with the cellular organization of the hypothalamus, leading to persistent alterations of the reproductive axis.

- The epigenetic, molecular and cellular organization of the GnRH network is most vulnerable to EDCs during early development.

- Effects of EDCs are not limited to classical agonist or antagonist action on sex steroid receptors but also induce long lasting gene expression as well as epigenetic changes in the developing brain.

- The study of low doses complex mixtures is required to mimic real world situations and better relate animal model studies to epidemiological data.

ABSTRACT

The onset of puberty and female ovulatory cycle all represent important developmental milestones of the reproductive system. They are controlled by a tightly organized network of neurotransmitters, neuropeptides as well as (epi)genetic and hormonal factors, which ultimately drive the pulsatile secretion of Gonadotropin-Releasing Hormone (GnRH). They also strongly depend on organizational processes that take place during fetal and early postnatal life. Therefore, exposure to environmental pollutants such as Endocrine Disrupting Chemicals (EDCs) during critical periods of development can result in altered brain development, delayed/advanced puberty and long-term reproductive consequences such as impaired fertility. While the gonads and peripheral organs are targets of EDCs, it has recently appeared that the organization of the neuroendocrine control of reproduction is also sensitive to environmental cues and disruption. Among other mechanisms, EDCs are known to interfere with the action of steroidal and non-steroidal receptors, and to alter enzymatic, metabolic as well as epigenetic pathways during development. In this review, we will discuss the cellular and molecular consequences of perinatal exposure to representative EDCs with a focus on the neuroendocrine control of reproduction, pubertal timing and the female ovulatory cycle mostly in rodents.

1. INTRODUCTION

Endocrine disrupting chemicals (EDCs) are substances capable of inadvertently interfere with endocrine systems by affecting hormone secretion or their targets, impacting populations and individuals given their ubiquity in our environment[1,2] (Table 1). There is a large variety of EDCs including drugs, pesticides and fungicides, plasticizers, industrial by-products and some naturally occurring botanical chemicals[2]. While humans and wildlife have coexisted with natural EDCs, man-made chemicals are a significant concern to public health due to their actions on cancer development, thyroid function, metabolism and obesity, cardiovascular function, and male and female reproduction[3]. Although the gonads have long been considered the major reproductive target of EDCs[4,5], recent data underline their impact on the developing brain (Figure 1), especially on Gonadotropin Releasing Hormone (GnRH) neurons. This subset of neurons located in the anterior hypothalamus secrete GnRH, which through its pattern of release, triggers a cascade of hormone-dependent processes orchestrating reproductive function. The hypothalamic control of gonadal function depends on organizational processes that take place during fetal and early postnatal life, a period sensitive to the effects of EDCs. GnRH neurons originate in the nasal placode during embryonic development and travel to their final destination in the hypothalamus [6]. Once in place, the activity of GnRH neurons is tightly controlled by a neuroendocrine network of neurotransmitters, glial derived growth factors, neuropeptides, (epi-)genetic and hormonal factors[7,8]. In mammals, GnRH release increases shortly during early postnatal life, in a period called "mini puberty". In rodents, a similar period of enhanced Follicle Stimulating Hormone (FSH) release around postnatal day 12 is responsible for the development of preantral ovarian follicles[9–11]. During infancy, the gonadotropic axis is quiescent until puberty, when the GnRH pulse generator is reactivated and therefore increases LH pulsatility starting adult reproductive life[12,13]. While males show a continuous production of sperm and gonadal

hormones, females show regular ovulatory cycles until the ovarian follicular reserve is depleted and the individual enters reproductive senescence or menopause[14].

The programming of pubertal maturation is finely tuned by sex steroids[15,16] and highly sensitive to early environmental factors[17]. EDC exposure during the perinatal or prepubertal period is associated with early or late pubertal onset in both boys[18–20] and girls[21–25] and later disruption of the ovarian and reproductive function as polycystic ovarian syndrome (PCOS) or early menopause[26,27]. In rodents, environmental toxicants affect the central control of puberty (reviewed in [17]) and ovulation as shown for the pesticide DDT[28,29], the plasticizer BPA[30–36], the polychlorinated biphenyls Arochlor 1254[37], the phytoestrogen genistein[38,39], the alkylbenzene p-tert-octylphenol[40], among others. Abnormal pubertal activation caused by EDCs may result from alterations taking place at the level of the hypothalamic GnRH network, the pituitary gland or the gonads themselves. In this review, we will analyze the cellular and molecular effects of EDCs on the hypothalamic control of puberty and reproduction focusing on the GnRH network throughout development, from early fetal development to adulthood. Regarding adulthood, we will prioritize *in vivo* female rodent models of developmental EDC exposure as the preovulatory LH surge provides a paradigm to study disruption of ovulation programming.

2. THE DIRECT EFFECTS OF EDCs ON GnRH NEURONS.

GnRH neuron [41, 42,43] migration from the olfactory placode (Figure 2) depends on fibroblast growth factor and prokineticin signaling, as well as Anosmin-1 interaction with the extracellular matrix[44–46]. GnRH neurons derived from nasal explants are considered well-differentiated[47] (equivalent to E18.5 mice GnRH neurons [48]) and appear to express ERβ[49,50], GPR30 and EERγ transcripts and/or protein [50–52]. Estrogen receptors in these neurons might play a role in sexual differentiation of the brain which takes place around that time (reviewed in [53]) and be a potential target for EDCs. Acute BPA at 0.5 and 50μM, comprising the

estimated circulating levels following exposure to the lowest-observed-adverse-effect (LOAEL)[54,55], directly inhibits intracellular calcium movement, a measure of GnRH neuronal activity in nasal placodes [52]. This BPA action is independent of GPR30 or EERγ and could involve voltage gated sodium, potassium and calcium channels, all present in GnRH neurons (reviewed in [56]) and targeted by BPA [57,58]. A crucial aspect of studies focusing on E2 or EDC effects *in vitro* is their concentration. It is assumed that GnRH neurons are exposed to picomolar levels of E2[59] even though such concentrations could be increased by local aromatase activity[60]. However, many studies have used pharmacological concentrations of estrogens[59,61–63], rendering them inadequate to extract accurate conclusions with physiological meaning. Thus, it is crucial to study EDC concentrations reflecting environmental exposure.

After their birth in the nasal placode, GnRH neurons migrate through the olfactory bulb, into the preoptic area during fetal life[41,48,64]. Early exposure to EDCs affects neuronal migration[65,66] and could lead to abnormal puberty and reproduction. Oral exposure of pregnant mice to ethinylestradiol at environmental and pharmacological doses (0.01, 0.1 and 1µg/kg/day) during GnRH neuron migration (E11.5-13.5) increases the number of GnRH neurons along the migratory path without affecting their distribution[65]. In contrast, maternal exposure to 2µg/d of BPA from E10 to P10 transiently decreases GnRH neuron number in pubertal male rats[66]. While these studies suggest that EDCs affect the number of neurons reaching the preoptic area, it is unknown whether they affect GnRH precursor proliferation, differentiation or migration directly through ERβ or GPR30 expression early in life[50–52], or affect other cell types involved in their migratory path.

Mature GnRH neurons convey the estrogen negative feedback onto the reproductive axis in both sexes. These cells express ERβ[49], GPR30[67], ERRγ[52] and STX-sensitive membrane ER[68]. While many EDCs such as BPA have relatively low affinity for ERα and ERβ[69], they have higher affinity for GPR30[67] and

ERRγ[70] and thus, could interfere with the action of E2 on those membrane receptors (Table 2). The use of immortalized GnRH neurons (i.e. mice GT1-7) provide insights into the direct effects of EDCs on postmigratory GnRH neurons, as they recapitulate their morphology and secretory capacity. However, certain limitations or differences need to be considered, like the expression of ERα by GT1-7 cells, not present in mature GnRH neurons. Using these cells, it was determined that the stimulatory effects of a mixture of polychlorinated biphenyl (Aroclor 1221) on GnRH transcripts and neuron morphology[37] are independent of classical ERα or β signaling. Moreover, using transgenic GnRH-GFP mice, it was demonstrated that genistein directly induces persistent membrane depolarization of GnRH neurons[71].

While most of the studies so far have focused on EDCs with estrogenic action, there is a lack of information on the effect of other widespread compounds (i.e. fungicides, herbicides, UV-filers) on GnRH neurons development and function.

3. THE EFFECT OF EDCs ON THE NEURONAL NETWORK CONTROLLING GnRH FUNCTION

Once GnRH neurons are in place, pulsatile GnRH release depends on an upstream network of neurons and glial cells that provide excitatory and inhibitory transsynaptic inputs to GnRH neurons (Figure 2). Neurotransmitters and neuropeptides, in particular GABA, glutamate, Kisspeptins, Neurokinin B, Dynorphin and RFamide peptides play a critical role in the regulation of GnRH secretion[72–74]. Glial cells impose a stimulatory effect to the GnRH neuronal network through secretion of prostaglandins and growth factors[12,75–77]. EDCs have been found to alter inhibitory and stimulatory inputs to GnRH neurons (Table 2). Still today, there is a complete lack of information about EDC actions on the glial cells involved in the stimulatory control of GnRH secretion.

3.1. GABAergic inputs to GnRH neurons

Although most GnRH neurons are excited by GABA, the effect of GABA is likely determined by the balance between $GABA_A$ and $GABA_B$ receptors in their soma and dendrites (reviewed in [78]). GABA plays a role at different developmental stages of the hypothalamic-pituitary-gonadal axis. First, it is involved in the regulation of GnRH neuron migration[79,80]. Later on, a decrease in GABAergic tone is crucial for the reactivation of the GnRH machinery at puberty[81,82], and appears to control the switch in estradiol feedback action in adult females[83]. Studies showed that early EDC exposure affects GABAergic input onto GnRH neurons[30,84–86]. Neonatal exposure to a very low dose of BPA (25ng/kg/day) delays pubertal maturation and diminishes GnRH secretion through increased hypothalamic GABAergic neurotransmission, whereas a high dose of BPA (5mg/kg/day) advances puberty through reduced GABAergic tone[30], in a typical non-monotonic dose-response[87]. Perinatal exposure of dams to high doses of BPA in drinking water (2.5mg/kg/day) has been shown to decrease GnRH release while increasing hypothalamic GABAergic tone[84], however, the effects on puberty onset was not studied. Although doses and periods of exposure differed, these two studies indicate that EDCs such as BPA directly affect GABAergic neurotransmission onto GnRH neurons. A study using ^{1}H-nuclear magnetic resonance metabolomics in the whole brain of animals exposed to a very low dose of BPA[85,86] from gestational day 8 (G8) to postnatal day 6 (PND6), showed a diminution of transaminases involved in the synthesis and consumption of L-glutamate, the primary substrate for GABA, further supporting the findings observed in the hypothalamus[86]. Other studies have shown that gestational exposure to mixtures of PCBs in rats alters GABA receptor expression in the POA and thus potentially the GnRH system receptivity to GABA[88]. Notably, the effects variy with the nature of the PCBs and sex, indicating a potential impact on the normal development of this sexually dimorphic nucleus[71].

3.2 Glutamatergic inputs to GnRH neurons

Glutamate plays a crucial role in activating GnRH neurons, in particular at the time of puberty[89,90]. While GnRH neurons express AMPA, NMDA and kainate receptors, the site of glutamate action within the GnRH neuronal network remains to be completely understood[91]. Large populations of glutamatergic neurons in the hypothalamus express ERα and are involved in the transmission of the estrogen feedback[92]. Neonatal exposure to the estrogenic insecticide dichlorodiphenyltrichloroethane (DDT) or estradiol leads to early acceleration of GnRH secretion, increased glutamate evoked GnRH release and early vaginal opening in female rats possibly trough genomic and nongenomic mechanisms[28,93]. The rapid effects of DDT on glutamate evoked GnRH release involved ER, aryl hydrocarbon receptor, and AMPA receptors[29]. On the other hand, gestational and lactational exposure to a high dose of BPA (2.5mg/kg/d) decreases serum LH and testosterone associated with decreased hypothalamic release of GnRH and glutamate *in vitro* in prepubertal male rats[94]. These studies suggest that glutamatergic neurons mediate some of the effects of EDCs on the activation of GnRH secretion around puberty[28,29].

3.3 Kisspeptin inputs to GnRH neurons

During the last twenty years, substantial effort has been made to identify hypothalamic factors responsible for the pubertal activation of the GnRH neurons and their regulation during adulthood. Mutations causing hypogonatropic hypogonadism and pubertal failure have been identified in genes that encode for the *KISS1* gene and its cognate receptor GPR54[95] or *TAC3* and its receptor *TAC3R*[96]. *Kiss1,* the gene that encodes for kisspeptins, plays a crucial role in the reactivation of the GnRH pulse generator at puberty[95,97]. In rodents, there are two main populations of kisspeptin neurons, the ARC and the AVPV kissppeptin neurons[98]. Kisspeptin neurons in the ARC in rodents are involved in GnRH activation around puberty[99,100]. The Kisspeptin neuron population in the AVPV is significantly larger in females compared to males[101]

and is involved in the preovulatory surge of LH[102]. Seventy percent of AVPV kisspeptin neurons express ERα[103], making them a target for gonadal steroids[104] and therefore vulnerable to EDCs (reviewed in [105]). Several studies[31,106–108] indicate that kisspeptin expression around puberty is sensitive to early exposure to EDCs, but the causal link between the changes in kisspeptin expression and abnormal puberty onset remains to be elucidated. Initial studies showed that neonatal exposure to estradiol benzoate[106], DES[107] or genistein[108] leads to decreased *Kiss1* expression in the hypothalamus of juvenile rats[106, 107] and kisspeptin immunoreactive fibers of adult female rats[108]. Very recent data suggest that the two main populations of kisspeptin neurons could have divergent sensitivity to EDC exposure[31]. Mice exposed to low doses of BPA from GD11-PND8, exhibit a persistent, but divergent, impairment of Kiss1 neuronal maturation, with more kisspeptin cells in the AVPV but consistently fewer kisspeptin neurons and lower *Kiss1 and Tac2* expression in the ARC[31]. Altogether, these data suggest that developmental exposure to EDCs could distinctively disrupt the normal differentiation of both kisspeptin populations ultimately affecting pubertal timing and ovulatory capacity. Additionally, pubertal exposure to BPA through direct infusion into the median eminence in rhesus monkeys, suppresses kisspeptin secretion and GnRH release in mid- to late-puberty[109], indicating that the pubertal period could be another window of sensitivity of the kisspeptin system to EDCs. Neonatal or prepubertal exposure to other EDCs such as dibutyl phthalate[110] or the mycotoxin zearalenone[111]also advances puberty and increases kisspeptin mRNA expression in the ARC[110] or whole hypothalamus[111] of female rats. Although conducted with high doses, these studies add to the bundle of evidence identifying ARC kisspeptin neurons as potential targets of EDC-induced alterations of pubertal timing.

3.4 Other factors controlling GnRH neurons

Only one publication links the effect of EDCs on RFamide-relatedpeptide-3 (RFRP3, a.k.a. NPVF or GnIH) inhibitory neuronal pathway[112–114] onto GnRH

neurons, perhaps explaining alterations in pubertal onset. Female rats exposed to 10μg/kg E2 or 50μg/kg BPA show decreased RFRP-3 fiber density and contacts on GnRH neurons and advance pubertal development[115].

Although perinatal exposure to a low dose of BPA disrupts the hypothalamic feeding circuitry by reducing NPY, POMC and AgRP projections into the ARC and paraventricular nucleus (PvN)[116,117], still today there is no information about the association between EDCs actions on the GnRH network and disruption in this feeding circuit.

4. EFFECTS OF EDCs ON THE POSITIVE OESTROGEN FEEDBACK

Reproductive maturity in females is characterized by the rise in GnRH network sensitivity to the estrogen positive feedback leading to the preovulatory LH surge and ovulation (reviewed in [118]). As ERα appears to mediate this positive feedback[119] but few or no GnRH neurons express this receptor[120,121], it is widely accepted that the AVPV population of kisspeptin neurons relay the estradiol positive feedback to GnRH neurons[98,122]. This specific population of kisspeptin neurons, is organized neonatally by sex steroids[106,123,124]. Male mice harboring a mutation which prevents membrane estrogen receptor alpha (mERα) signaling[125], show increased number of kisspeptin neurons in the AVPV, suggesting an active feminization of this kisspeptin population by estradiol. Even more importantly, female mice neonatally treated with estradiol benzoate[125] or testosterone[126] have a profile of kisspeptin expression similar to males and are unable to evoke a positive E2 feedback after neonatal androgenization[126]. Therefore, during prenatal life, ERα appears to play a predominant role in the masculinization of the AVPV kisspeptin neurons, but during postnatal life it is involved in feminization[127,128]. This makes AVPV kisspeptin neurons a preferential target explaining the effects of EDCs on the preovulatory surge of LH.

While there is a vast literature documenting the ovarian effects of EDCs, we will focus exclusively on the neuroendocrine effects of EDCs leading to abnormal estrous cycle and ovulation (Table 3). Gestational or early postnatal exposure to EDCs disrupts the programming of spontaneous[39] or induced LH surge[33–36,40], leading to abnormal cyclicity. A 3 or 4-day neonatal exposure to a high dose of genistein[38], estradiol benzoate or PTT, an ERα agonist[129] decreases the density of AVPV kisspeptin fibers projecting to GnRH neurons in adult female rats, affecting ovarian morphology and inducing an anovulation-like phenotype[38,108]. Genistein and other phytoestrogens have a relatively high affinity for ERβ[130], suggesting a dual action, they can directly alter GnRH neurons as they express ERβ, and/or AVPV ERα and ERβ kisspeptin expressing neurons or the ARC ERα kisspeptin expressing neurons[122]. While neonatal exposure to 50 µg or mg of BPA/kg (PND1-4) do not affect AVPV kisspeptin fibers in adult female[129], oral exposure to lower doses during gestation and early postnatal life (G11 to PND8) increases the number of kisspeptin neuron cell bodies and fibers in the AVPV of pubertal female mice, indicating that exposure to low environmental doses of BPA affects the organization of this specific population[31]. This underlines the importance of testing environmentally relevant exposures at sensitive periods of development. However, the impact of such an increase in kisspeptin neurons on the preovulatory surge and ovulation has not been studied and remains to be documented. Because early postnatal estrogens appear to decrease the kisspeptin neuronal population in females[125], this suggests that BPA might act through non-estrogenic pathways under this circumstance. Estrogenic EDCs are not the only ones that interfere with the central hypothalamic mechanisms of ovulation. In mice, a 4 month developmental exposure to perfluorooctane sulfonate (PFOS) decreases AVPV Kiss1 mRNA expression during proestrus, suppressing preovulatory LH surge and disrupting antral and preovulatory ovarian follicles[131]. This effect seems to be mediated by reduced ovarian E2 output caused by PFOS disruption of the steroidogenic acute regulatory (StAR) protein, since normalization of E2 levels

counteracts PFOS effects[131]. Moreover, the PCB mixture A1221 is also able to interfere with the preovulatory LH surge after germ-cell exposure in rats[132]. In this study, while only a small effect in LH concentration was detected during proestrus after *in utero* exposure to A1221, the next generation, affected through germ-cell exposure, did not show LH or progesterone peak at proestrus[132]. Altogether, these studies clearly show that early exposure to EDCs interferes with the responsiveness of the GnRH network to the positive estrogenic feedback required for the preovulatory LH surge.

The pubertal period appears to be another window of sensitivity for the programming of ovulation. For example, exposure to organochloride pesticides between PND21-23 advances the onset of puberty and alters estrous cyclicity[133]. A continuous infusion of BPA, about tenfold higher than the human concentrations documented in biomonitoring studies, reduces LH pulse frequency in prepubertal sheep[134].

Gestational and/or postnatal exposure to EDCs has been shown to affect ERα and β transcript and protein expression in the AVPV[135–141] and ARC[33,138,142] (table 2), thereby affecting neuronal sensitivity to endogenous sex steroids as well as EDCs themselves. No study so far has examined the effects of EDCs on ER expression in specific cell population such as the kisspeptin neurons.

There has been growing evidence of the action of EDCs through non-hormonal mechanisms, challenging their classical estrogenic/antiandrogenic action[143–147]. Clock genes in the AVPV play a key role in the control of circadian signals timing the preovulatory surge of LH[143,144]. This mechanism seems to be independent of estrogen levels[144] and vulnerable to endocrine disruption. *In vitro* BPA exposure alters Bmal1 and Per2 mRNA expression[145] in immortalized hypothalamic neurons. Similarly, adult exposure to 25ng/kg/d BPA for 15 days decreases Per1 mRNA expression in the MBH and impairs the preovulatory LH surge, estrous cyclicity and late ovarian follicle development[146]. While the non-estrogenic regulation of AVPV kisspeptin secretion through the action on

circadian genes seems to be mediated by noradrenaline signaling[147], there is not enough information linking BPA with this mechanism.

5. THE EFFECT OF EDCs ON THE EPIGENETIC CONTROL OF THE GNRH NETWORK

Epigenetic processes affect gene expression without changes in DNA sequence, they include DNA methylation, usually at the carbon-5 position of cytosines in CpG dinucleotides, histone post-translational modifications (PTMs) that affect chromatin packaging[148] or non-coding RNA involved in mRNA degradation and half-life[148] (Figure 3) (i.e. micro-RNAs, pico-RNAs, small nuclear-RNAs and long non-coding RNAs). This epigenetic mode of control is a relay of environmental information to the gene networks controlling physiological processes[149], including the GnRH system[150]. More importantly, epigenetic changes not only produce long term effects in somatic cells, but also affect the germline, inducing alterations that are inherited transgenerationally[151–154].

5.1 Epigenetic changes in somatic cells

During the last several years, a significant number of studies shed new light into how epigenetic processes affect sexual differentiation (reviewed in [155,156]) and the onset of puberty[157,158]. DNA methylation, histone PTMs and ncRNAs play a crucial role in the regulation of the transcriptional machinery of neurons involved in reactivating the GnRH pulse generator around puberty[7,157,159]. In the rat ARC, the 5' regulatory region of the Kiss1 gene contains both repressing and activating epigenetic regulators. This particular chromatin landscape allows for rapid activation and/or repression of gene expression. Before puberty, the Kiss1 promoter is enriched in the repressing histone H3 trimethylated at lysine 27 (H3K27me3), induced by the presence of the Polycomb Group (PcG) of epigenetic silencers[160]. As puberty approaches, the Trithorax Group (TrxG) of epigenetic activators counteracts these repressive marks by imposing activational H3K4me3 and H3K27Ac PTMs at the ARC Kiss1 promoter and

enhancer regions respectively[157]. After completion of puberty, the ovary dependent activation of the AVPV Kisspeptin neurons during the preovulatory surge of LH requires increased histone H3 acetylation at the *Kiss1* promoter/enhancer region in an estrogen-dependent manner[159]. Moreover, the H3K27 demethylase JMJD3 has been shown to control female puberty and ovulation by regulating AVPV *Kiss1* gene expression in an E2 dependent fashion[161]. EDC exposure during development could affect DNA methylation, histone PTM or non-coding RNAs (ncRNAs) expression (Table 4). Initial studies looking at the epigenetic effects of EDCs in the brain identified changes in DNA methyltransferase (DNMT) expression[162–165] or methylation changes at specific loci[166,167]. While most of these studies identify changes in mRNA expression of key enzymes involved in CpG methylation or histone PTMs, EDCs could also affect the levels of co-factors involved in their activity, especially S-adenosylmethionine (SAM). Although, there is still a complete lack of information on how EDCs affect cellular levels of substrates and co-factors involved in the enzymatic epigenetic processes at the GnRH network, much needs to be learned from studies on other models. It is known that S-adenosylmethionine (SAM) is a methyl donor for practically all methylation reactions within a cell. SAM is produced from dietary methionine by a methionine adenosyltransferase (MAT), depletion of SAM leads to a global reduction in methylation capacity[168]. *In vivo* exposure to 3-methyocholanthrene, a polycyclic arylhydrocarbon, reduces liver MAT mRNA and protein levels[169] in adult rats, while BPA exposure in the agouti mouse model induces DNA hypomethylation through SAM depletion, and is reversed by diet supplementation with folic acid[170]. While histone methylation is dependent on the cellular levels of SAM and flavin adenine dinucleotide (FAD), a co-factor of some lysine demethylases, histone acetylation is dependent on cellular levels of Acetyl-CoA (a co-factor for histone acetyl transferases) and nicotinamide adenine dinucleotide (NAD+), a cofactor for the Sirtuin family of deacetylases[168].

Very few studies have reported *in vivo* effects of EDCs on histone PTMs in the brain. BPA and phthalates alter chromatin structure in primary cortical neuron cultures[171], neuroblastoma cell lines[172] and other tissues[173] and perinatal BPA exposure persistently increases histone H3 acetylation in the cerebral cortex and hippocampus of postnatal male mice[174]. However, no study so far has reported effects of EDCs on histone PTMs in the neuronal networks controlling GnRH release.

It is known that miRNA expression in the mPoA is sexually dimorphic and sensitive to perinatal estrogens[175]. BPA affects miRNA expression in the ovary[176], testis[177] and adipocytes[178] and some miRNAs and their transcriptomic responses appears significantly associated with the presence of persistent organic pollutants in the blood of healthy subjects[179]. Only one study has shown that a short gestational exposure to a PCB mixture leads to an increase in the expression of several miRNA (mir-219, mir-132, mir-7, mir-145, let-7a) in the female mPoA around puberty but a decreased expression in miRNAs in the male hypothalamus at adulthood[175]. However, few target genes were affected with such exposure. The similarities between the effects of the PCB mixture and estradiol benzoate in females suggests that the mechanisms that underlie this increase in miRNA expression is of estrogenic nature.

While most of the aforementioned epigenetic effects of EDCs are associated with their classical agonist or antagonist action on sex steroid receptors and thus, gene expression, more research needs to be done to understand how fluctuations in cellular metabolites affect the epigenetic machinery in the GnRH network and how different EDCs interfere with this process.

5.2 Epigenetic changes in germ cells

Early intrauterine exposure to environmental stressors leads to long lasting epigenetic and phenotypic changes, including the risk of disease, throughout generations. This transgenerational inheritance is defined as the transmission

of a biological trait to the subsequent generations through the germ line. In an exposed gestating F0 female, the F1 embryo and F2 generation germ line are exposed (multigenerational inheritance), whereas the F3 and subsequent generation are not directly exposed (transgenerational inheritance). Although there is a lack of information on how embryonic programming affects adult phenotypes, epigenetic transmission of traits is most likely the mechanism by which the genome gains flexibility when under environmental pressures.

Multigenerational studies have provided evidence of the persistent effects of EDCs on reproduction[132,153,180,181]. Gestational exposure to Aroclor 1221 suppressed LH and progesterone levels in rats exposed while *in utero,* a phenotype more severe in the offspring, suggesting that alterations in the hypothalamic gene network is transmitted to F2 animals[132]. EDC effects appear to be transmitted through the germline. For instance, exposure to vinclozolin[153,180,181] affects mate preference or stress response and alters mRNA expression of Methyl Binding Domain 2 (*Mbd2)* or DNA Methyl Transferase 3b (*Dnmt3b)* in the rat central amygdala, two genes associated with DNA methylation, in the F3 generation[151,162]. *In utero* BPA exposure also produces transgenerational effects on social recognition and activity[154] in mice, in part due to transgenerationally increased number of ERα expressing cells in the female AVPV, potentially through alterations in germ cell DNA methylation[182].

EDC exposure also leads to transgenerational effects on pubertal timing[125–128], but the epigenetic pathways conveying information from a wide range of stimuli to the hypothalamic neurons regulating the onset of puberty stills need to be elucidated. Maternally expressed gene 3 (*Meg3)*, an imprinted lncRNA associated with precocious puberty[187] and neurobehavioral problems[188], is increased in the mPoA of F3 juvenile males[189] exposed to BPA. However, DNA methylation of this gene was not affected by BPA[189], suggesting that other epigenetic mechanisms, such as histone PTMs, could explain the transgenerational alteration of *Meg3* expression.

Although expensive and time consuming, transgenerational studies are needed to identify the effect of different EDCs on the GnRH network in order to understand how life-long exposure to low doses of EDCs or EDC mixtures affect reproductive development and fertility throughout generations, especially in view of the secular trend of timing of puberty[17] and fertility[190] in humans.

6. CONCLUSIONS

While the gonads have long been considered as the main targets of EDCs, recent years have brought evidence regarding the sensitivity of the neuroendocrine control of reproduction to environmental disruption. Environmental factors influence such processes through different mechanisms depending on whether they take place during perinatal life, puberty or adulthood. There is a growing body of evidence linking *in utero* and early life exposure to EDCs with different disorders, especially disorders of puberty and fertility. Although it has become clear that some EDCs act through disruption of the GnRH neuronal network affecting estradiol responsiveness or directly altering gene expression in upstream neuronal neurons in charge of regulating GnRH release, there is a lack of consistency in the research approach used i.e. dosage, time and route of exposure. It is essential to focus on EDC exposures that mimic real life situations seen in human populations, meaning low doses over a prolonged period of time. This inevitably takes us also to the fact that in real life situations, humans are simultaneously exposed to a wide variety of EDCs at very low doses instead of single chemical exposure over a very restricted time period. The aforementioned research demonstrates that if environmental exposures take place during a critical period for GnRH system development, it is expected to produce alterations in GnRH response, estrous cyclicity and ovulation. Data summarized here shows that EDCs can directly interact with membrane or nuclear receptors and interfere with the early organization of the circuitry controlling GnRH neurons during puberty and ovulation. ARC and AVPV

kisspeptin neurons play a crucial role in the control of puberty and ovulation respectively and are sensitive to environmental factors. Other hypothalamic genes involved in sensing peripheral changes in metabolism such as the leptin receptor are known to be permissive cues for puberty onset and ovulation and are affected by gestational exposure to EDCs[141]. This underlines the importance of exploring the effects of EDCs on the crosstalk between the hypothalamic control of energy balance and reproduction.

In addition to neurons, astrocytes and tanycytes, which participate in the pubertal and preovulatory activation of GnRH secretion (reviewed in [191]), could be targeted by EDCs as suggested by *in vitro* studies showing that estradiol and nonylphenol alter glial morphology in hypothalamic primary cultures[192]. In another study, gestational exposure to BPA increases the number of microglial cells and the expression of proinflammatory mediators in the embryonic hypothalamus, possibly participating in neurotoxicity[193]. Thus, further research is needed to better understand the effects of EDCs on glial function.

Additionally, if germ cells are exposed to EDCs, it may yield to epigenetic reprogramming of the offspring, increasing the risk of disease in the most sensitive tissues in multi- and transgenerational fashion. It is highly important to identify these windows of sensitivity to EDC action in order to be able to design preventive treatments in order minimize the impact on the affected subjects and to block the propagation of disease to future generations.

Finally, recent data have proven the crucial role of epigenetic regulation affecting the transcriptional activity of neurons involved in stimulating GnRH release[157]. However, much remains to be done to identify the pathways conveying epigenetic information from environmental disruptors to the hypothalamic cells controlling pubertal onset and ovulation. This review provides evidence that EDCs alter gene expression and change phenotype in part by modifying the epigenome, especially when exposed at crucial periods of life. The characterization of gene expression profiles, as well as chromatin landscapes associated with the genes susceptible to EDCs, will ultimately

pinpoint epigenetic biomarkers of EDC exposure in the GnRH network. These epigenetic biomarkers could be used as early diagnostics of adult disease or to identify preventive measures before disease symptoms develop. Nowadays, with the development of single cell technologies, we are in a privileged position to understand how environmental cues affect epigenetic trajectories of small and hard to isolate cellular populations like the GnRH neurons. Moreover, it is highly desirable to identify peripheral markers that inform of neuronal epigenetic landscapes and gene expression changes induced by exposure to different EDCs.

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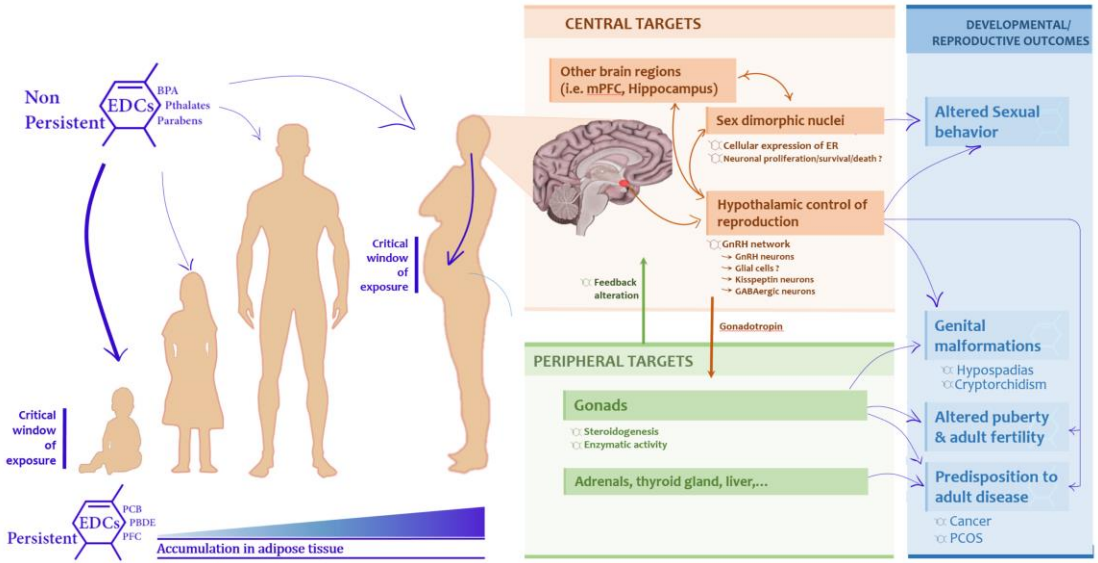

Figure 1. Ubiquitous presence of persistent and non-persistent Endocrine Disrupting Chemicals (EDCs) in the environment and consumer products has an impact on human health, particularly during critical windows of development. Persistent compounds are known to accumulate in adipose tissue throughout life, transfer to the fetus through the placenta and accumulate in maternal milk. Non persistent compounds are metabolized quicker but also transfer through the placenta. EDCs activate hormonal and non-hormonal receptor pathways leading to alterations in hormonal balance, epigenetic modifications and alterations of sex steroid metabolism. Such actions impact the brain and the gonads. In the brain, the development of sexual dimorphic regions and the hypothalamic control of puberty and ovulation are especially vulnerable to EDCs. Ultimately, such alterations caused by EDCs could lead to abnormal puberty, infertility and behavioral disorders and increased predisposition to adult disease.

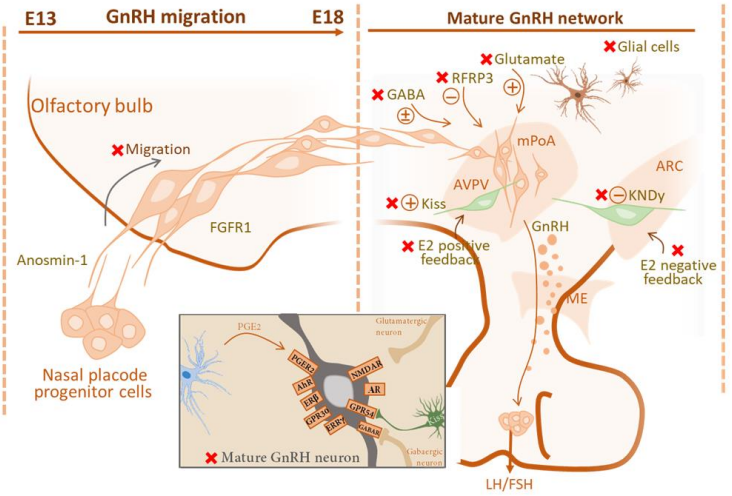

Figure 2. GnRH neurons originate from nasal placode progenitors cells around embryonic day 13 in the rat and migrate through the cribriform plate and across the olfactive bulb to reach the preoptic area at embryonic day 18. Migration guidance is orchestrated by olfactory axons and influenced by factors as anosmin-1 and fibroblast growth factor and prokinetic signaling. Once GnRH neurons reach the mPoA a serie of transsynaptic and glial cells conformate the GnRH neurons. Factors such as glutamate, GABA, kisspeptin and RFRP3 regulate pulsatile GnRH secretion during puberty and ovulation. ARC Kisspeptin neurons (KNDy) play an essential role in the control of the estrogen negative feedback by interacting with the GnRH nerve terminals, whereas kisspeptin neurons of the AVPV regulate the GnRH positive feedback. GnRH neurons themselves express a wide array of receptors potentially targeted by EDCs. Known and potential targets of EDCs are marked with a red cross. RFRP3: RFamide-related peptide 3, GABA: gamma aminobutyric Acid, Kiss: kisspeptin, FGFR1: fibroblast growth factor receptor 1, AVPV: anteroventral periventricular, mPoA: median preoptic area, GnRH: gonadotropin releasing hormone, KNDy: kisspeptin, neurokinin B, and dynorphin expressing neurons, ME: median eminence, LH: luteinizing hormone, FSH: follicle stimulating hormone, PGE2: prostaglandin E2, AhR: aryl hydrocarbon receptor, ERβ: , NMDA: n-methyl-d-aspartate, GPR54: kisspeptin receptor, ERRγ: estrogen-related receptor gamma.

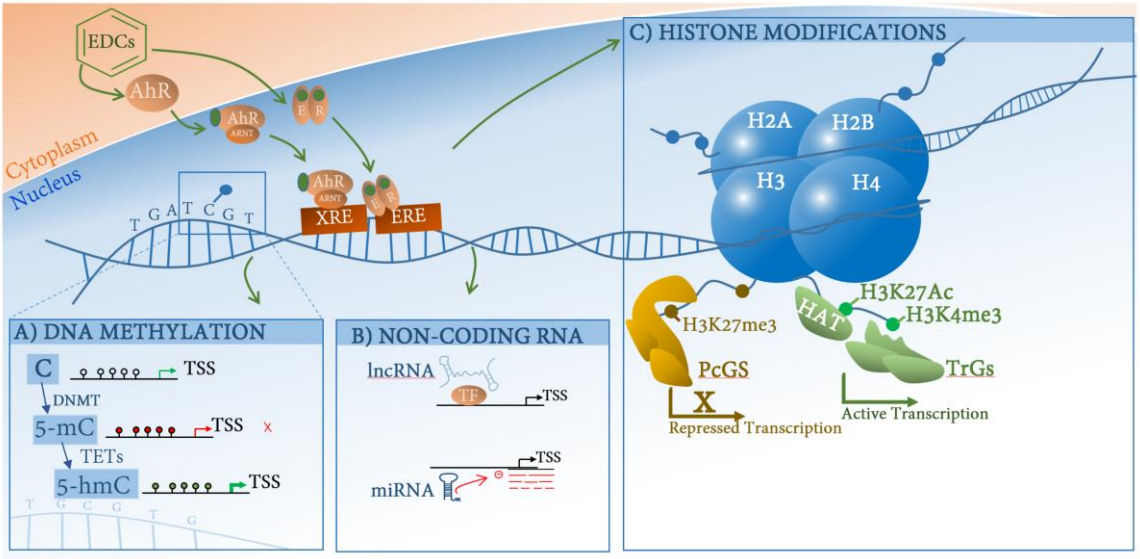

Figure 3. Epigenetic mechanisms targeted by EDCs in the brain. EDCs can alter nuclear receptor recruitment at specific loci (i.e. ERE, RXR) and modify DNA methylation, non-coding RNA expression or histone PTMs. (A) CpG methylation is catalyzed by the action of DNA methylatransferases (DNMT) leading to the production of 5-methylcytosine (5-mC). Such reaction can be reversed by the ten-eleven translocation family of proteins (TETs). (B) EDCs can alter the expression of long (lncRNA), small (sncRNA) and micro (miRNA) non-coding RNAs. Non-coding RNAs are known to interact with specific epigenetic modifiers and direct DNMTs to specific loci. (C) Post-translational histone tail modifications (i.e. methylation, acetylation, etc.) regulate chromatin state, inducing or repressing gene transcription. Chromatin remodeling proteins, such as histone acetyltransferases (HAT), the polycomb (PcGs) or trithorax (TrGs) group of proteins, catalyze such chemical modifications and have been recently shown to be involved in pubertal onset[136].

Table 1. Sources of endocrine disrupting chemicals

Category	**Examples**	**Sources / Uses**
Phthalates	Di(2-ethylhexyl) phtalate, diisobutyl phthalate,dDibutyl phthalate	Plasticizer, consumer products (i.e. cosmetics, dental material)
Bisphenols	Bisphenol A, bisphenol F, bisphenol S	Plasticizer (PVC and epoxy resins), food containers, building material
Parabens	Methylparaben, butylparaben, propylparaben	Consumer producs (i.e. cosmetics, personal care)
Pesticides / fungicide	Triclosan, atrazine, methoxychlor	Agriculture runoff, vegetables
UV-Filter	4-methylbenzylidene camphor, octyl methoxycinnamate, 3-benzylidene camphor	Sunscreens and cosmetics
Pharmaceutical	Diethylstilbestrol, acetaminophen, ethinylestrol	Contraception, analgesic/antalgesic
Flame retardants	Polybrominated diphenyl ethers (i.e. BDE-47, BDE-99), tetrabromobisphenol A	Furniture, dietary, carpet backing
PCBs	Aroclor mixtures (i.e. 1254, 1248, 1016)	Coolants, insulating fluids, lubricanting oils, building material additives
Phytoestrogens	Genistein, coumestans	Soybean, sesame seeds, oats
Dioxins and dibenzofurans	2,3,7,8-Tetrachlorodibenzo-p-dioxin	Industrial by-product
Alkylphenolic compounds	Propylphenol, nonylphenol, octylphenol	Detergents, fuel and lubrificant additives
Perfuoroalkyl compounds	Perfluorooctanoic acid, perfluorooctanesulfonic acid	Repellents and coating for cookware, textiles and carpets

Table 2. Cellular and molecular targets of EDCs in rodent GnRH network.

Target	Sex	Results	EDC	WoE	References
ERα mPoA	F	↑	BPA BPS Mix MXC	G G+P	194 195 196 197
		↓	BPA 4-MBC	P G+P	198 199
	M	↓	4-MBC	G+P	198
ERα AVPV	F	↑	BPA DBP	P G+P	137 138 182
		↓	BPA PCB	P G+P	136 138 141
ERα ARC	F	↑	BPA	G	138
		↓	BPA	P	33
	M	↑	BPA	G	138
		↓	DEHP Mix	G	177 194
ERα VMH	F	↑	BPA	G	138
		↓	4-MBC BPA	G+P	198
	M	↑	BPA	G	138
		↓	4-MBH	G+P	198
ERβ mPoA	F	↓	BPA MXC	G G+P	136 196
ERβ ARC	F	↓	DEHP	G	178
ERβ AVPV	F	↓	BPA PCB	P G+P	136 140 142
	F	↑	GEN BPA	P	139 31
	M	↑	BPA	G	138
		↓	BPA	P	142
Kisspeptin AVPV	F	↑	BPA PCB	G G+P	31 164 200
		↓	BPA DES GEN	P	107 108 138 201
	M	↑	BPA	G+P	66
Kisspeptin mPoA	M	↓	PCB Mix	G	141 194
Kisspeptin ARC	F	↓	BPA GEN	P G+P	31 115 108
	M	↓	DEHP Mix	G	177 194
GABA MBH	F	Alt	PCB	P	174
		↑	BPA	P G+P	171 202
		↓	BPA	P	202
	M	↓	BPA	G+P	172
RFRP3 MBH	F	↓	BPA	P	178
Glutamate MBH	F	↑	DDT	P	20 202
	M	↓	BPA	G+P	21
TAC2 ARC	F	↓	BPA	G+P	31

Notes: 4-MBC: 4-Methylbenzylcathinone, BPA: bisphenol A, BPS: bisphenol S, DBP: Diethyl phthalate, DEHP: Bis(2-ethylhexyl) phthalate, DES: Diethylstilbestrol, MXC: Methoxyhclor, PCB: polychlorinated biphenyl, WoE: Window of exposure, G: gestational exposure, P: early postnatal exposure,

↑: *increased*, ↓: *decreased*.

Table 3. Effects of EDCs on GnRH and the hypothalamic control of ovulation.

Target	Results		EDC	WoE	References
GnRH	↑	mRNA	BPA MXC	P G+P Ad	33 34 197
		Secretion	4-NP BPA DDT MXC	P	28 30 107
	↓	mRNA	BPA MXC PFOS TBT	G P Ad	33 146 196
		Secretion	BPA DES DPN EQ GEN OMC PPT	P	40 107 109 203
Responsiveness	↑		GEN	P	204
to GnRH	↓		ZEA	P	204
LH surge	Altered		ATR BP GEN MXC PFOS TBT	P G+P	34 36 40 131 132 205

Notes: 4-NP: 4-nonylphenol, ATR: atrazine, BP: butyl paraben, BPA: bisphenol A, DDT: diethyl-dichloroethane, DES: Diethylstilbestrol, DPN: diarylpropionitrile , EQ: equol , GEN: genistein, MXC: methoxyhclor, PFOS: Perfluorooctanesulfonic acid, PPT: Propylpyrazole triol, TBT: tributyltin, OMC: Octyl methoxycinnamate, ZEA: Zearalenone, WoE: Window of exposure, G: gestational exposure, P: early postnatal exposure, Ad: adult exposure, ↑: *increased*, ↓: *decreased*.

Table 4. Effects of EDCs on DNA methylation, non-coding RNA and histone posttranslational modifications (PTMs) in the brain.

	Target	Effect	EDC	WoE	Sex	Region	Specie	Age	Ref.
DNA Methylation									
Enzymes	*Dnmt1*	Alt	BPA	G	F M	PFC & MBH	Mice	P28	166
		↑	BPA	G+P	M	CTX & HIP	Mice	P21	174
		↑	BPA	G+P	F	AMY	Rat	P45	206
		↑	BPA	G+P	M F	CTX	Mice	Adult	207
		↓	BPA	G+P	M	CTX & HIP	Mice	Adult	174
		Alt	A1221	G	F	AVPV	Mice	P28	164
	Dnmt3a	Alt	BPA	G	F M	PFC & MBH	Mice	P28	166
		↑	BPA	G+P	M	CTX	Mice	P21	174
	Dnmt3b	↓	BPA	G+P	M	MBH	Rat	Adult	167
		↓	Vin	G	F	VMN	Rat	F3 Adult	162
	5-mC	↓	BPA	G+P	M	CTX & HIP	Mice	P21 Adult	174
Methylation	*Esr1*	↓	EB	G+P	F	MBH	Rat	Adult	197
		↑	BPA	G	M	PFC	Mice	P28	166
		↑	BPA	G	F	MBH	Mice	P28	166
	Bdnf	↑	BPA	G+P	F	HIP	rat	Adult	167
	Mbd2	↑	VIN	G	F	CeA	Rat	F3 Adult	162
	Grin2b	↓	BPA	G+P	F	HIP	Rat	Adult	208
Non-coding RNA									
lncRNA	*Meg3*	↑	BPA	G	M	MBH	Mice	F3 P28	189
miRNA	*mir-7/132/145/219, let-7a*	↑	A1221	G	F	mPoA	Rat	P30	175
	mir-9/124a/132/145/219, let-7a	↓	A1221	G	M	mPoA	Rat	Adult	175
Histone PTMs									
Enzymes	*Hdac2*	↑	BPA	G+P	M	CTX	Mice	P21 Adult	174
		↑	BPA	G+P	M	HIP	Mice	P21	174
		↓	BPA	G+P	M	HIP	Mice	Adult	174
Histone PTMs	*H3K9ac*	↑	BPA	G+P	M	CTX & HIP	Mice	P21 Adult	174
	H3K14ac	↑	BPA	G+P	M	CTX & HIP	Mice	P21 Adult	174

Notes: WoE: Window of exposure lncRNA: long non coding RNA, miRNA: micro RNA, Dnmt: DNA methyltransferase, 4-NP: 5-methylcytosine, Esr1: estrogen receptor 1, Bdnf: brain-derived neurotrophic factor, Mbd2: methyl-CpG binding domain protein 2, Meg3: maternally expressed 3, Hdac2: histone deacetylase 2, Alt: altered, ↑: *increased.*, ↓: *decreased.*BPA : $bisphenol A$, $A1221$: $aroclor1221$$G$: $gestational$, P : $postnatal$, PFC : $prefrontalcortex$, MBH : $mediobasalhypothalamus$, CTX : $cortex$, HIP : $hippocampus$, AMY : $amigdala$, $AVPV$: $anteroventralperiventricularnucleusofthehypothalamus$, CeA : $centralamygdala$, $mPoA$: $medianpreopticarea$.

Appendix B

Review Two

Endocrine disruptors and possible contribution to pubertal changes

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Endocrine disrupters and possible contribution to pubertal changes

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The onset of puberty strongly depends on organizational processes taking place during the fetal and early postnatal life. Therefore, exposure to environmental pollutants such as Endocrine disrupting chemicals (EDCs) during critical periods of development can result in delayed/advanced puberty and long-term reproductive consequences. Human evidence of altered pubertal timing after exposure to endocrine disrupting chemicals is equivocal. However, the age distribution of pubertal signs points to a skewed distribution towards earliness for initial pubertal stages and towards lateness for final pubertal stages. Such distortion of distribution is a recent phenomenon and suggests environmental influences including the possible role of nutrition, stress and endocrine disruptors. Rodent and ovine studies indicate a role of fetal and neonatal exposure to EDCs, along the concept of early origin of health and disease. Such effects involve neuroendocrine mechanisms at the level of the hypothalamus where homeostasis of

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reproduction is programmed and regulated but also peripheral effects at the level of the gonads or the mammary gland.

Introduction

Puberty represents a crucial milestone in one's reproductive life. For this reason, any effect of the environment on pubertal timing might announce later consequences on reproduction. For the last 20 years, data has accumulated suggesting changes in pubertal timing and a possible role for exposure to endocrine disrupting chemicals (EDCs). This review will summarize the recent data regarding secular trends in age at puberty in boys and girls as well as the likely increase in central precocious puberty incidence in girls. Finally, we will review the epidemiological and animal data suggesting a role for endocrine disrupting chemicals in the reported changes in pubertal timing.

Secular trend in pubertal timing in girls

Because menarcheal age and breast development are clinically easier to assess, data regarding female puberty is significantly more abundant than male data.

A reduction in menarcheal age had been reported in European countries [1] as well as in the United States [2] between 1890 and 1960. An overall advancement in female pubertal timing that averaged 4 years in a century was derived from those observations [2]. This evolution is considered to be due to the improvement of socioeconomic conditions and nutritional status. The hypothesis is consistent with the Frisch and Revell theory according to which a critical amount of body fat is necessary in order to enter puberty [3]. Since 1960, the secular advancement in menarche has appeared to cease in countries such as Belgium [2,4]. In the United States, menarcheal age remained stable, around 12.7–12.9 years, between 1973 and 1997 [5–7] but a recent longitudinal study has reported a slightly lower median age at menarche (12.2 years) in New-York, Ohio and California [8]. A similarly moderate decrease in age at menarche was reported in Danish cohorts between 1991 and 2006 (13.42 and 13.13 years, respectively) [9]. Interestingly, a stronger secular decrease persists in emerging countries such as India or China [10,11]. However, an extensive spatial heterogeneity regarding age at menarche still persists in those regions of the world.

Starting in the nineties, several large US studies have reported a persistent secular decrease in age at onset of breast development in girls compared to the mid-20th century. A publication from the American Academy of Pediatrics reported a mean age at B2 of 10 years in White American girls and 8.9 years in African-American [7]. These findings generated comments on the possible overestimation of breast development because assessment was made through visual inspection only, whereas palpation may be required to distinguish between adipose and glandular tissue. The Third National Health and Nutrition Examination Study (NHANES III), a population-based study conducted between 1988 and 1994, confirmed the findings and found the median age of thelarche to be 10.4 years in white Americans and 9.5 in black Americans [12]. Following those observations, in 2007, an expert panel stated that the data were sufficient to suggest a trend toward earlier breast development in the United States over the second half of the 20th century [13]. A more recent longitudinal study by Biro et al. observed a continuous decrease in age at B2 in American girls aged 6–8 years at enrollment [14]. Mean age of breast stage 2 was 8.8 years for black, 9.2 years for Hispanic, 9.6 for non-Hispanic white, and 9.9 years for Asian participants, illustrating the influence of race/ethnicity on breast maturation. Notably, body mass index had a greater effect on age at menarche than did race and ethnicity. Those data are consistent with data collected in Scandinavian countries: The Copenhagen Puberty Study reported an advance of nearly one year for attainment of Tanner stage 2 between girls from the 1991 cohort and girls from the 2006 cohort [9]. Recent European studies have also highlighted the existence of a non-Gaussian distribution of the age at B2 in girls with a distribution skewed to the left, meaning that more girls start puberty early than late. A recent longitudinal study in Greek girls showed a median age at B2

of 10 years with the 25th and 75th centiles being 9.2 and 10.6 years, respectively and a skewness of −0.44 [15]. Cross-sectional data in Belgium [4] were consistent with those findings: the distribution of age at B2 was skewed towards earliness whereas the distribution for age at menarche was skewed towards lateness. The negative distortion of age at B2 appears to be more marked in recent studies [4]. These observations suggest that during the last decades of the 20th century, some girls tended to enter puberty earlier and some to end puberty later, which was not reflected by changes in median ages. Very recently, Woelfle et al. assessed secular trends on pubertal development in more than 7000 patients with Turner syndrome [16]. They found a decline in the age at spontaneous thelarche of about 2 years between those born before 1980 and those born in 2000–2004. The prevalence in spontaneous puberty onset appeared to increase between 1980 and 1995–1999. As in healthy girls, one can speculate that the increase in weight SDS and exposure to EDCs explains such secular trend in pubertal timing.

The secular changes in age at pubertal onset and changes in distribution pattern indicate a potential role for environmental factors. However, the causal relationship is extremely difficult to establish. When searching for possible causes and mechanisms of changes in pubertal timing, the default hypothesis is hypothalamic-pituitary maturation. Peripheral mechanisms, however, can coexist with central mechanisms or secondarily facilitate them. Such a concept is supported by the dissociation between advancement in age at onset of breast development in Denmark, the Netherlands, Belgium or the United States without parallel change in menarcheal age. Two different pubertal events can respond differently to hormonal and EDC influences. Moreover, a single pubertal event can be influenced by different endocrine pathways. For instance, breast development can be due to ovarian estrogen secretion under the stimulation by pituitary gonadotropins and/or estrogenic effects associated with exposure to EDCs. The interpretation of the mechanistic role of sex steroids or related environmental factors is complex due to the multiple sites where they can interact between the hypothalamus and the peripheral target tissues. Interestingly, there was no difference in serum follicle stimulating hormone and luteinizing hormone between the two Danish cohorts in 1991 and 2006 which would suggest that the change in timing of breast development would not result from an early activation of the pituitary-gonadal axis. However, multiple animal models indicate that early exposure to EDC can alter the hypothalamic programming of puberty (see below).

The role of the obesity epidemics in puberty timing deserves to be developed as well. Because nutritional status is known to be a determinant of puberty, a causal relationship between obesity and earlier onset of puberty has been hypothesized. Evidence has accumulated that a sufficient amount of fat mass signaling to the neuroendocrine system through leptin is a prerequisite to the onset of puberty. Leptin can stimulate pulsatile gonadotropin-releasing hormone (GnRH) secretion and is indeed necessary but not sufficient to account for onset of puberty [17]. It also appears that both energy balance and pubertal timing share common regulatory factors that could be jointly influenced. Notably, in Biro's study, body mass index (BMI) was the strongest predictor of earlier age at breast development [8]. In their study, white non-Hispanic participants with BMI <85th percentile had similar age at breast development as white girls in 1997 [7], indicating that the secular trend had plateaued in that group. Other studies have suggested an association between body weight and puberty timing. Adiposity rebound (BMI increase between 2 and 8 years) has been linked with earlier growth spurt in Danish boys and girls [18]. Aksglaede et al. have found that BMI at 7 years is a significant predictor of age at pubertal growth spurt in a cohort of more than 150,000 boys and girls [18]. However, secular trend towards early onset of puberty is found in all BMI categories suggesting involvement of other factors than peripubertal weight.

Secular trend in pubertal timing in boys

Pubertal timing in boys being relatively more difficult to assess, the number of available studies is significantly lower. In the 1990s, data collected from the American population-based study NHANES III suggested an advance in age at onset of genital development [12,19,20]. However, due to the lack of reliable data from the previous population based study NHESIII [21], no conclusions could be made regarding the trend in pubertal onset in boys. Only a limited number of secular trend studies exist in Europe, and they did not support a secular trend toward earlier age at pubertal onset in boys between the 1960s and the 1990s [22,23]. Recent data collected in Scandinavian countries are consistent with an

earlier pubertal timing: the age at attainment of testicular volume above 3 ml was about 3 months earlier in the 2006–2008 cohort compared to the 1991–1993 cohort [24]. Significantly higher LH, but not testosterone, levels were found in 2006–2008 compared to 1991–1993 and BMI Z-score increased significantly from 1991 to 1993 to 2006–2008. However, pubertal onset and LH levels were no longer significantly different between study periods after adjustment for BMI.

Recently, the Puberty Cohort in Denmark (2012–2017) has reported a mean age at Tanner Stage 2 of 11.1 years [25] corresponding to an advance of around half a year compared to the previous Danish cohort [24]. Interestingly, age at pubertal endpoint (Tanner stage 5) was similar. It is important to note that the methods for data collection differed, making the comparison between the two studies difficult: the first study used clinical examination whereas the second one used questionnaires with self-reported information on Tanner stages. Another milestone of male puberty, voice break, has been reported to advance as well. The Puberty Cohort in Denmark (2012–2017) reported an average age of 13.1 years, which is considerably earlier than former studies reporting between 15.5 and 14.0 years in the period 1968 to 2005 [26,27]. Here again however, the evaluation methods differ, rendering comparison difficult to conduct.

As already pointed out in girls, the initial pubertal signs and signs of completion of puberty appear to show divergent secular changes, suggesting heterogeneity in response of pubertal events to modulating factors. As an example, the first 3–10% of boys with evidence of initial pubertal increase in testicular volume ($\geq$4 ml) are younger than in the past (reviewed in 4), whereas the final 3–10% (centile 90 and 97) appear to attain adult testicular volume ($\geq$15 ml) later (reviewed in 4).

Is prevalence of precocious puberty increasing?

Data regarding the prevalence of precocious puberty and the existence of a secular trend is very scarce. In 2005, an epidemiological study based on national registries in Denmark estimated that 0.2% of Danish girls and <0.05% of Danish boys had some form of precocious pubertal development [28], which was much higher than data from 40 years ago [29]. However, the overall prevalence could be overestimated because precocious pubertal development included patients with true central precocious puberty as well as patients with premature adrenarche and premature thelarche. Moreover, the age limit of 8 years and 9 years for diagnosis of sexual precocity in girls and boys respectively was extended to 9 and 10 years. Several years later, another study in Denmark found an increase in the number of girls referred for early pubertal development between 1993 and 2009 [30]. In this cohort of 449 Caucasian girls, 88 girls (19.6%) were diagnosed with central precocious puberty (breast development before 8 years and LH peak in the LHRH test > 5 U/L or LH/FSH ratio > 0.66 or basal LH > 0.3 U/L) and only six with peripheral precocious puberty. It follows that more than 50% of the girls referred for advance of pubertal development had no true precocious puberty but conditions that need only a follow up without treatment (Tanner Breast 2 between 8 and 9 years, premature thelarche or premature adrenarche). Another report from tertiary care centers with pediatric endocrinology in Spain estimated a lower incidence compared with Danish data since the prevalence for girls was 0.037% and <0.0005% for boys [31].

European data seem to be consistent with observations in other part of the world such as in Korea. In an epidemiologic study using a national registry, the annual incidence of central precocious puberty increased steeply, in particular, among girls between 2004 and 2010 [32].

Interestingly, this increase in central precocious puberty appears to be more marked in girls than boys [31–33] which could suggest a greater sensitivity of female subjects to environmental factors.

Pubertal timing and EDC exposure in humans

Except in conditions such as industrial spills resulting in accidentally high exposure of a given population to a given EDC, demonstrating the effects of endocrine disruptors on pubertal timing in human conditions remains difficult for several reasons. Girls and boys entering puberty are exposed to low doses of hundreds of chemicals, rendering causation difficult to demonstrate. The exposure may have taken place during the prenatal or the early postnatal period leading to a long latency between exposure and the potential consequences on pubertal timing. Moreover, the effects of exposure to

endocrine disruptors can differ depending on the studied endpoint (i.e. breast development or menarche).

The possible involvement of early life exposure to endocrine disruptors on pubertal timing has been suggested by the observation that the risk of sexual precocity was 80 times higher in children migrating to Belgium for international adoption compared to Belgian native children. Those children had been formerly exposed to the estrogenic insecticide dichlorodiphenyltrichloroethane (DDT) in the country of origin [34]. Vasiliu et al. [35] have also reported early menarche after presumable prenatal/early postnatal exposure to dichlorodiphenyldichloroethylene; a metabolite of DDT. In Denmark, similar findings have been reported in the daughters of women exposed to pesticides due to occupation in green houses [36]. Prenatal exposure to pesticides very early during gestation was associated with earlier breast development in girls. This association appeared not to be caused by changes in gonadotropins, but rather to higher androgen levels, which indirectly may increase oestrogens through aromatization. Studies focusing on the effects of soy-based infant formula on pubertal development have led to mixed results. Most studies are retrospective and limited in term of participants. Some studies have reported that age at menarche appeared to be earlier in girls fed with soy products during infancy [37,38]. In a contemporary British cohort, *in utero* exposure to phytoestrogens was associated with changes in age at menarche with opposite effects depending on the measured phytoestrogen [39]. Prenatal exposure to flame retardants such as polybrominated diphenyl ethers (PBDEs) has been associated with later age at menarche [40] while the National Health and Examination Survey (NHANES) showed that higher serum PBDE concentrations between ages 6 and 8 years [41] or 12 and 19 years [42] was associated with slightly earlier menarche or older age at onset of breast development respectively, illustrating once more the potential importance of the window of exposure and sensitivity.

Data about effects of early exposure to endocrine disruptors on pubertal timing in boys are sparse and sometimes contradictory. In 2005, Hsu and colleagues reported a decrease of testosterone levels and increase in FSH levels in chinese boys of mother accidentally exposed to high doses of polychlorinated biphenyls/polychlorodibenzofuranes by ingestion of rice oil contaminated by those compounds [43]. In a birth cohort from the Faroe Islands, prenatal exposure to PCB was associated with lower serum concentration of LH and testosterone [44]. However, only the neonatal levels were predictive of slightly smaller testes [44]. Those results were similar to those described by Eskenazi and colleague [45] in the CHAMOCOS cohort: lower LH and testosterone values were observed in boys of mothers with higher maternal DDT levels. Concomitantly lower LH and testosterone levels in serum suggested a central origin of delayed puberty.

Mechanisms of changes in pubertal timing caused by EDCs

The secular trend in age at onset of puberty as well the possible increased incidence in central precocious puberty in girls suggest a role for environmental factors, EDCs in particular. However, these epidemiological findings in humans face limits as far as clarification about which chemical is involved, which age window is critical, and where the mechanisms take place in the hypothalamic-pituitary-gonadal axis. Therefore, human studies need to be complemented by studies using animal models.

It appears that the programming of pubertal maturation is an adaptive mechanism responding to environmental factors very early on. In primate and ovine species, pubertal timing is sexually dimorphic, and sex steroids play a crucial role in gender differences in pubertal timing. Thus it appears logical that early alteration of sex steroid action could affect the programming of puberty timing. The effects of environmental changes or stressors on pubertal timing depend on the period of occurrence or exposure. For instance, prepubertal underfeeding leads to delayed puberty; overfeeding and excess of adiposity in humans may lead to early puberty, whereas intrauterine growth restriction is associated with early puberty (reviewed in 4). What do rodent models teach us regarding critical periods of sensitivity to EDCs? In female rats, for instance, the effect of bisphenol A on pubertal onset appears to depend on the timing of exposure. Vaginal opening is unchanged after gestational exposure, whereas early postnatal exposure is followed by early puberty [46]. Exposure of male rodents to EDCs such as DDE [47], vinclozolin [47] or diethylstilbestrol [47,48] leads to delayed puberty after postnatal exposure (postweaning) as opposed to absence of effects after prenatal exposure (reviewed in 4). The

developmental variations in rodents' sensitivity to EDCs, however, cannot be strictly extrapolated to the critical periods in humans on the account of possible differences between species.

The interpretation of the mechanisms of action of environmental factors is complex due to the multiple sites where they can interact between the hypothalamus and the peripheral target tissues. Abnormal pubertal development caused by EDCs may result from alterations taking place at different levels: the hypothalamic GnRH network, the gonadotropic cells or the gonads themselves (Fig. 1). The physiological acceleration of GnRH secretion before puberty in rat hypothalamic explants can be used to study the effects of environmental factors on the hypothalamic control of puberty [49–52]. In order to model early exposure of migrant children to the pesticide dichlorodiphenyltrichloroethane (DDT), neonatal female rats were exposed to DDT from postnatal day 6–10. Such exposure led to advanced acceleration of pulsatile GnRH secretion and early onset of puberty [53]. The effect involved estrogen receptors, the dioxin/aryl hydrocarbon receptor (AhR) and a subtype of glutamate receptor [54,55]. Using the same model, we showed that neonatal exposure to an environmentally relevant dose of BPA (25 ng/kg/d) for 15 days was followed by a delay in developmental reduction of GnRH interpulse interval studied *ex vivo* on postnatal day 20. In contrast, exposure to BPA 5 mg/kg/d for 15 days resulted in a premature reduction in GnRH interpulse interval and a trend toward early vaginal opening [56]. Notably, the very low and environmentally relevant dose of BPA delayed neuroendocrine maturation related to puberty through increased inhibitory GABAergic neurotransmission [56]. Recent studies have shown that other members of the GnRH network could be targeted (Fig. 1). The ontogeny and function of kisspeptin neurons are profoundly influenced by gonadal steroids and vulnerable to endocrine disruption [57]. In addition, alterations of sensitivity to sex steroids in sexual dimorphic regions of the hypothalamus can have long-lasting consequences on the control of puberty and reproduction. It has been shown that EDC can affect the expression of estrogen receptors and alter the sensitivity of specific brain regions to endogenous estrogens [58] or polybrominated diphenyl ether-99 [59] and, by consequence, may alter GnRH secretion. EDCs can also interfere with the physiological (prominently inhibitory) feedback mechanisms of sex steroids in hypothalamic-pituitary function, while they can also act as a primer of neuroendocrine maturation [55]. Finally, they can interfere with hormones at the level of peripheral target tissues. Breast development could result from estrogenic EDC effects independent of the hypothalamic-pituitary maturation. This dissociation of effects on breast development and menarche could account for the secular reduction in the correlation coefficient between the ages at occurrence of the two pubertal events reported by some authors [60].

Recent data has shown that female puberty is controlled by epigenetic mechanisms such as histone modifications, DNA methylation and non-coding RNA [61]. Environmental factors such as EDCs affect epigenetic regulation in several tissues but studies identifying how epigenetic pathways convey information from EDCs to hypothalamic neurons regulating the onset of puberty are still required.

Summary

Recent data have shown a persistent secular trend toward early pubertal onset in girls and boys. Detailed analysis actually reveals that the pattern of age distribution is affected. Current variations in pubertal timing involve a trend towards negative or positive distortion for initial or final pubertal stages, respectively, both in girls and boys. This suggests some heterogeneity in response of pubertal events to modulating factors. Those subtle changes are important in clinical practice since the extreme lower and upper age limits in the normal population are used to define early or late maturation. Relatively scarce data suggest that the incidence of central precocious puberty is increasing as well. This trends appears more marked in girls, which again suggest a sexual difference in sensitivity to environmental factors.

The changes taking place in the borderline physiological timing of puberty can be of clinical relevance due to behavioral consequences. Early puberty has been shown to be associated with more frequent adolescent risk taking behaviors and with psychopathologies persisting throughout adult life [62–65]. These issues may account for a significant impact on both the clinician and the society. In addition, altered puberty timing can be a marker of subsequent reproduction abnormalities later in life. In the long term, early and precocious timing of puberty also predict a slight but significant increase in the risk of breast cancer [66], angina, hypertension and type 2 diabetes [67].

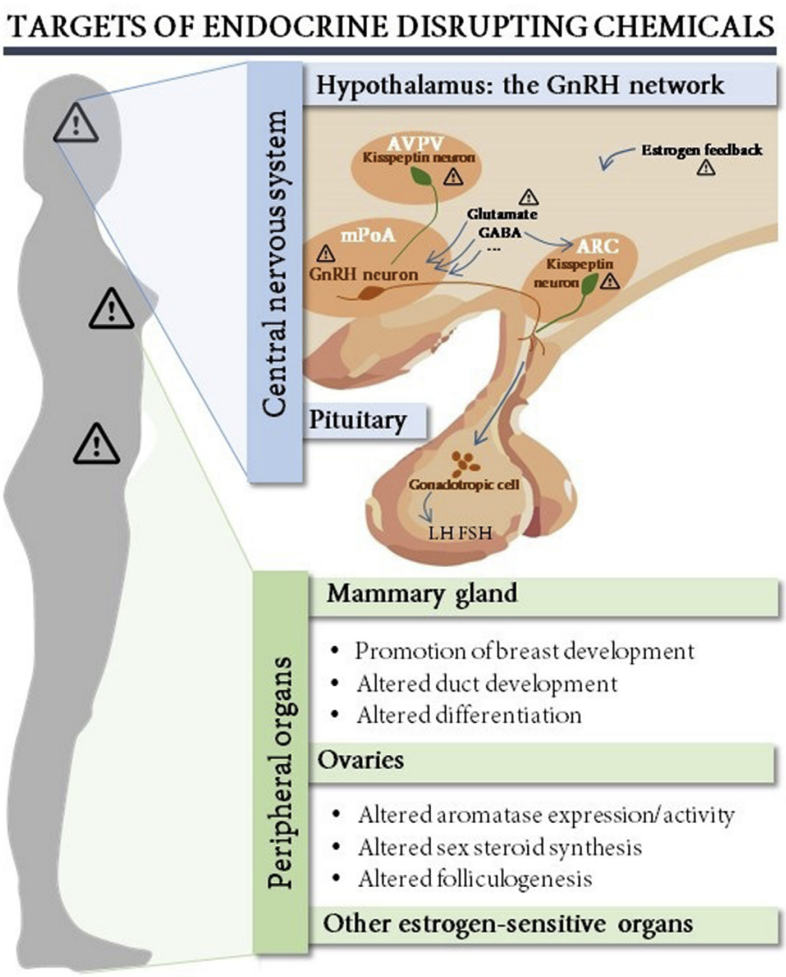

Fig. 1. Schematic representation of EDC targets potentially involved in alterations of pubertal development. Abnormal pubertal development caused by EDCs may result from disruption taking place at different levels: the hypothalamic GnRH network, the gonadotropic cells, the gonads themselves or the mammary gland. ⚠ represent the potential targets of EDCs as identified by animal studies.

Epidemiological and animal studies have shown that the programming of puberty and reproduction is very sensitive to exposure to EDCs. Both hypothalamic and gonadal targets have been identified. However, the Organisation for Economic Co-operation and Development currently recommends to use the postnatal days 22–42 as validated exposure period for EDC testing. Those guidelines ignore the earlier period that is critical for neuroendocrine maturation, particularly the developmental acceleration of pulsatile GnRH secretion and miss the concept that pubertal timing can be influenced long before the period immediately preceding the onset of puberty. Moreover, in recent years it has been shown that female puberty is controlled by epigenetic mechanism as histone modifications, DNA

methylation and non-coding RNA. Epigenetic repression and activation of gene transcription is a core mechanism by which epigenetics regulates pubertal development. EDCs can affect epigenetic regulation in several tissues and may convey information from a wide range of stimuli to hypothalamic neurons regulating the onset of puberty.

Practitioners can play an important role in both collecting and providing information about the potential burden of EDCs. They should be involved in the promotion of a consumer behavior reducing that burden because puberty is only one among several health issues related to EDCs such as abnormal sexual differentiation, neurodevelopmental diseases, metabolic syndrome and increased risk of neoplasia.

Conflicts of interest

The authors have no conflict of interest to declare.

Practice points

- Age distribution of pubertal signs points to a skewed distribution towards earliness for initial pubertal stages and towards lateness for final pubertal stages. It is thus very important to take both the beginning and end of puberty into account when evaluating secular trend in pubertal timing.
- Recent data indicate an increased incidence of central precocious puberty but this data requires larger epidemiological studies.
- Measurement of EDC exposure in individual patient with precocious puberty cannot be interpreted reliably. However, the practitioner can play an important role in collecting data regarding the potential burden of EDCs or the documentation of an epidemics of abnormal puberty in a given region.
- The clinician can play an important role in the promotion of a consumer behavior reducing EDC burden in the pregnant woman and child.

Research agenda

- The effects of EDC mixtures on the control of puberty and reproduction need to be explored further.
- Recent data indicate that EDCs can affect puberty and reproduction transgenerationally. These concerning effects need to be investigated.
- Potential changes in central precocious puberty (especially in girls) incidence need to be documented.

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Appendix C

Experimental study One

Persistent vs Transient Alteration of Folliculogenesis and Estrous Cycle After Neonatal vs Adult Exposure to Bisphenol A

Persistent vs Transient Alteration of Folliculogenesis and Estrous Cycle After Neonatal vs Adult Exposure to Bisphenol A

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Exposure to bisphenol A (BPA), a ubiquitous endocrine-disrupting chemical (EDC), is known to produce variable effects on female puberty and ovulation. This variability of effects is possibly due to differences in dose and period of exposure. Little is known about the effects of adult exposure to environmentally relevant doses of this EDC and the differences in effect after neonatal exposure. This study sought to compare the effects of neonatal vs adult exposure to a very low dose or a high dose of BPA for 2 weeks on ovulation and folliculogenesis and to explore the hypothalamic mechanisms involved in such disruption by BPA. One-day-old and 90-day-old female rats received daily subcutaneous injections of corn oil (vehicle) or BPA (25 ng/kg/d or 5 mg/kg/d) for 15 days. Neonatal exposure to both BPA doses significantly disrupted the estrous cycle and induced a decrease in primordial follicles. Effects on estrous cyclicity and folliculogenesis persisted into adulthood, consistent with a disruption of organizational mechanisms. During adult exposure, both doses caused a reversible decrease in antral follicles and corpora lutea. A reversible disruption of the estrous cycle associated with a delay and a decrease in the amplitude of the LH surge was also observed. Alterations of the hypothalamic expression of the clock gene *Per1* and the reproductive peptide phoenixin indicated a disruption of the hypothalamic control of the preovulatory LH surge by BPA. (***Endocrinology*** **160: 2558–2572, 2019)**

Bisphenol A (BPA) is a ubiquitous endocrine-disrupting chemical (EDC) used in the production of polycarbonate plastics and epoxy resins (1). Despite its partial ban in some countries, it is currently one of the most largely used chemical compounds in the world with >8 billion tons produced each year (2). Human exposure is nearly universal in developed countries and occurs mainly through contaminated beverages and food (3). Several studies indicate widespread contamination of fetuses and neonates, leading to the questions as to whether such an EDC can affect development (4–6) and whether there is a limit for a safe exposure. Currently, the US Environmental Protection Agency "safety level" of BPA is set at 50 μg/kg/d, that is, 1000-fold the average human exposure (1). The European Food Safety Authority's tolerable daily intake was recently lowered to 4 μg/kg/d (7).

Sex steroids play a crucial role perinatally in "organizing" the control of female reproduction. For that reason, the adult female estrous cycle is altered following exogenous exposure to sex steroids during that vulnerable perinatal period (8). Therefore, the effects of early exposure to BPA on puberty and reproduction are a matter of concern. Recent evidence suggests that exposure to BPA during this sensitive developmental period could have long-term impacts on reproductive function (9). Early exposure to BPA affects puberty onset, with

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Abbreviations: AVPV, anteroventral periventricular nucleus; BPA, bisphenol A; CLARITY-BPA, The Consortium Linking Academic and Regulatory Insights on BPA Toxicity; EDC, endocrine-disrupting chemical; *Esr1*, estrogen receptor 1; *Esr2*, estrogen receptor 2; *Gad2*, glutamic acid decarboxylase 2; *Gat2*, glutamic acid transporter 2; IQR, interquartile range; MBH, medial basal hypothalamus; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases; NPY, neuropeptide Y; PND, postnatal day; PoA, preoptic area; rFSH, rat FSH.

effects depending markedly on the window and dose of exposure, and a possible nonlinear dose–response relationship (10–25). Effects of neonatal exposure to BPA on estrous cyclicity have produced inconsistent results. Whereas a few studies did not show any effect on estrous cyclicity (26–29), others reported abnormal cyclicity (12–14, 30–33). Prenatal or perinatal exposure to BPA also decreases the number of preantral follicles in mice (34) and rats (35) and leads to a decline in fertility and fecundity (36). Taken together, these studies suggest that prenatal or neonatal exposure to BPA doses in the milligram or microgram ranges affects several structures and functions of the neuroendocrine system and the ovaries. Whether the exposure to very low doses of BPA neonatally or during adulthood results in different effects on female neuroendocrine and ovarian functions remains largely unknown.

We have recently shown that neonatal exposure to a very low dose of BPA (25 ng/kg/d) delays the developmental changes in GnRH secretion before puberty, whereas a high dose of BPA (5 mg/kg/d) results in early occurrence of those neuroendocrine changes (15). This effect is followed by a delayed or advanced vaginal opening after exposure to the low or high dose of BPA, respectively. In this study, the aim was to elucidate whether such a neonatal exposure to a very low dose of BPA could produce persistent disruption of folliculogenesis and estrous cycle that could be consistent with disturbed organization. We also used the high BPA dose because opposing effects on GnRH secretion and pubertal timing were seen after using the low and the high doses of BPA neonatally (15). Finally, the aim was to evaluate whether adult BPA exposure in similar conditions would produce persistent or transient effects on ovulation and folliculogenesis.

Materials and Methods

Animal care and exposure

Adult female Wistar rats from the animal facility of the University of Liège were housed individually in standardized conditions (12-hour dark/12-hour light phase from 4:00 PM, 22.8°C, and food and water *ad libitum*). All animals were raised in BPA-free cages (polypropylene cages, catalog no. 1291H006, Tecnilab, Someren, Netherlands) and fed EDC- and phytoestrogen-free chow (V135 R/Z low-phytoestrogen pellets, Ssniff, Uden, Netherlands). Water was supplied in glass bottles.

Two timings of exposure were studied: female rats were exposed neonatally or during adulthood. Neonatal exposure to BPA started on postnatal day (PND) 1 and ended on PND 15. Animals received a daily subcutaneous injection (0.05 mL) of corn oil (vehicle) or one of the two doses of BPA, a low environmental dose of BPA of 25 ng/kg/d or a high dose of 5 mg/kg/d (BPA, catalog no. 239658; Sigma-Aldrich, St. Louis, MO). BPA was diluted in ethanol at an initial concentration of 100 mg/mL and then diluted in corn oil. The same concentration of ethanol was added to the control solution of corn oil. Once we obtained the final solution, tubes were opened to allow ethanol evaporation. Subcutaneous injections were given every 24 hours between 10:00 AM and 12:00 PM. Adult exposure took place from PND 90 to PND 105 under the same conditions. All experiments were carried out with the approval of the Belgian Ministry of Agriculture and the Ethics Committee at the University of Liège.

Experimental design

Effects of exposure to BPA from PND 1 to 15 on estrous cycle and folliculogenesis

Litters were homogenized for size and sex ratio on the first postnatal day of life to have 10 to 12 pups per litter and a 1:1 male/female ratio. Cross-fostering of a maximum two pups per litter was used when homogenization was required. The day of birth was considered as PND 0. Pups were weaned on PND 21. Twenty-eight female pups born from eight dams were exposed from PND 1 to 15 to 25 ng/kg/d (n = 7) or 5 mg/kg/d of BPA (n = 7) or corn oil (vehicle) (n = 14). The animals were followed for estrous cyclicity from the time of vaginal opening until PND 105 when they were euthanized to study ovarian folliculogenesis during the diestrus stage.

Effects of exposure to BPA from PND 90 to PND 105 on estrous cyclicity, GnRH and LH secretion, hypothalamic gene expression, and folliculogenesis

Eighty-one female rats were followed for estrous cyclicity from PND 60 onward. Among these females, only those (n = 74) showing at least three regular cycles out of four consecutive cycles at PND 90 were selected for the exposure experiment. These adult female rats were exposed to 25 ng/kg/d of BPA (n = 27) or 5 mg/kg/d (n = 26) or corn oil (n = 21) for 15 days from PND 90 to PND 105. A group of females in diestrus were euthanized 24 hours after the last dose of BPA or corn oil to measure plasma and pituitary LH and FSH levels (control, n = 9; BPA at 25 ng, n = 12; BPA at 5 mg, n = 7), hypothalamic gene mRNA expression (control, n = 5; BPA at 25 ng, n = 6; BPA at 5 mg, n = 5) as well as folliculogenesis (n = 6 per group). Only females in diestrus based on smear results were considered for analysis.

GnRH pulse frequency was analyzed *ex vivo* 24 hours after the last dose of BPA by using a hypothalamic explant incubation of females on diestrus (n = 4 per group). Another group of BPA-exposed female rats was followed for estrous cyclicity (BPA at 25 ng, n = 11; BPA at 5 mg, n = 11) until 4 weeks after the exposure. Among those females, a subgroup undertook serial blood samples to determine LH surge (BPA at 25 ng, n = 5; BPA at 5 mg, n = 5). Finally, a set of females was euthanized 4 weeks after exposure, and one ovary per animal was collected to study folliculogenesis on diestrus (control, n = 6; BPA at 25 ng, n = 6; BPA at 5 mg, n = 6).

Estrous cyclicity

Animals exposed from PND 1 to PND 15 were examined daily to evaluate estrous cyclicity from the day of vaginal opening until PND 105. Estrous cyclicity was measured with vaginal smears taken every day in the beginning of the afternoon as described previously (37). Rats exposed during adulthood were examined for estrous cyclicity from 2 weeks before the exposure to 4 weeks after the end of exposure to BPA.

We defined a regular cycle as a sequence of metestrus, diestrus, proestrus, and estrus in 4 consecutive days (38). The percentage of females having a regular cycle and the time spent in every stage of the cycle were calculated every period of 8 days corresponding to two full estrous cycles.

Hypothalamic explant incubation and GnRH assay

As previously shown, a neonatal exposure to BPA significantly affects GnRH pulse frequency at PND 20 (15). To determine whether GnRH frequency was affected after adult BPA exposure, GnRH secretion from hypothalamic explant was studied *ex vivo* 24 hours after the last subcutaneous injection of BPA. As previously described (39, 40), this method allows reliable measurement of GnRH pulsatility. Briefly, after decapitation, the brain was placed ventral side up. Two sagittal incisions along the lateral hypothalamic sulci and two transversal incisions of 2 mm were made 2 mm ahead from the anterior boundaries of the optic chiasm and along the caudal margin of the mammillary bodies. Then, the hypothalamic region including both the medial basal hypothalamus (MBH) and the medial preoptic area (PoA) were transferred into an individual chamber, in a static incubator, submerged in MEM. The incubation medium was collected and renewed every 7.5 minutes for a period of 4 hours. The GnRH released into the incubation medium was measured in duplicate using a radioimmunoassay method with intraassay and interassay coefficients of variation of 14% and 18%, respectively. The highly specific CR11-B81 (41) rabbit anti-GnRH antiserum (1:80,000) was provided by Dr. V. D. Ramirez (Urbana, IL) (42). Data below the limit of detection (5 pg per 7.5-minute fraction) were assigned that value.

Serum and pituitary LH and FSH radioimmunoassays

Blood samples and pituitaries were quickly collected 24 hours after the end of adult exposure to BPA or corn oil. Blood samples were stored overnight at 4°C, followed by decantation of serum and stored at −20°C until the assay was performed. Pituitary samples were stored in PBS at −20°C before homogenization with ultrasound and centrifugation to obtain the supernatant used for the assays.

Serum and pituitary LH and FSH levels were determined using a double Ab method and an RIA kit [mouse LH RIA, rat FSH (rFSH) RIA], supplied by the National Institutes of Health [Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Hormone and Peptide Program, Torrance, CA]. Antibodies used were NIDDK anti–rFSH-S-11 (43) and LH antiserum AFP-240580 (44). rFSH antigen NIDDK rFSH-I (AFP-5178B) and rat LH-I-8 (AFP-12066B) were labeled with ^{125}I by the chloramine-T method, and the hormone concentration was calculated using the mouse LH and rFSH reference preparation (LH, AFP-5306A; FSH, NIDDK rFSH-RP-2, AFP-4621B) as standard. The intraassay and interassay coefficients of variation were 6% to 9% and 7% to 10% for LH and FSH, respectively. The sensitivity of the assay was 4 pg/100 μL for LH and 0.125 ng/100 μL for FSH.

LH surge

Females were handled for habituation to tail-blood sampling daily during 2 weeks prior to the experiment. Pre-exposure and postexposure blood samples were collected 2 and 4 weeks before and after BPA exposure, respectively. During those periods, samples were collected every hour from 1:00 PM (3 hours prior to the beginning of the dark cycle) to 10:00 PM during proestrus, defined by vaginal smear. Because cyclicity was disrupted during BPA exposure, blood samples were collected from 11:00 AM to 10:00 PM during 2 consecutive days prior to the expected estrus stage during the second week of exposure and LH was measured using an ultrasensitive ELISA LH assay (45). Briefly, 96-well high-affinity binding plates (Corning Life Sciences, Corning, NY) were coated overnight with a bovine monoclonal antibody (46) (in 0.015 M Na_2CO_3 and 0.035 M $NaCO_3$ coating buffer, pH 9.6). Samples and serial dilution of known concentrations of LH were incubated for 2 hours. After incubation, a rabbit polyclonal primary antibody for LH (44) (1:10,000), a polyclonal goat anti-rabbit IgG secondary antibody (1:1000; Dako, Glostrop, Denmark), and 1-Step™ ultra tetramethylbenzidine ELISA substrate (Thermo Fisher Scientific, Waltham, MA) were added to each well. The sensitivity of this assay was 0.06 ng/mL and intraassay and interassay coefficients of variation were 6.3% and 10%, respectively.

For each detected pulse, the amplitude was determined by subtracting the highest LH value from the basal value immediately prior to the onset of the pulse. Overall basal levels of LH secretion were determined by combining a minimum of the five lowest LH measurements from each mouse.

Ovarian histology

When the ovaries were removed, they were weighted on PND 105, 90 days after the end of neonatal exposure to BPA or corn oil or 24 hours after the adult exposure to BPA. To determine whether folliculogenesis was affected by BPA, the ovaries were removed on PND 105 after neonatal exposure to BPA and either on PND 105 or PND 135, corresponding to 24 hours or 30 days after the end of adult exposure to BPA. The ovaries were fixed in 4% paraformaldehyde overnight, dehydrated in 70% EtOH, and paraffin embedded. For histological analysis, 8-μm coronal sections were cut using a microtome. Every other section was deparaffinized, stained with hematoxylin and eosin, covered with a coverslip, and examined for quantification of folliculogenesis.

For quantification, images of every other section throughout the whole ovary were acquired using an automated digital microscopy system DotSlide (catalog no. BX51TF, Olympus, Aartselaar, Belgium). DotSlide images taken at a magnification of ×10, which were in a proprietary format, were converted into a standard TIFF format and threefold decimated, which are easier to handle. Thereafter, quantification of follicles and corpora lutea was carried out manually with Aperio ImageScope v12.3.2.8013 software (SCR_014311; Leica Biosystems, Danaher Corporation, Wetzlar, Germany) by an experimenter blinded to treatment. Total ovarian volume was automatically calculated using an original program developed using the image analysis toolbox of the MatLab (SCR_001622, 2016a, MathWorks, Natick, MA) software.

Folliculogenesis was analyzed by quantifying follicles at every stage of folliculogenesis: primordial, primary, secondary, antral, and atretic follicles. Additionally, cysts and corpora lutea were identified. The follicles were classified according to well-established criteria (47, 48). Double counting of late-stage follicles was avoided by digitally marking each follicle throughout the consecutive images. Each follicle was counted once whenever the oocyte was present. For quantification of early stage follicles (primordial and primary follicles), a twofold correction factor was added to compensate for the sections that were not analyzed.

Measurements are expressed as number of follicles or corpora lutea per volume (cubic millimeters).

Real-time PCR

Because neonatal exposure to BPA altered the expression of hypothalamic genes involved in the GABAergic pathway [glutamic acid decarboxylase 2 (*Gad2*) and glutamic acid transporter 2 (*Gat2*)] (15), we studied the expression of those genes after adult exposure to corn oil or BPA in the MBH and PoA. The clock genes *Per1*, *Per2*, *Bmal1*, and *Clock*, as well as the reproduction-related peptide phoenixin (*Pnx*), the estrogen receptor 1 (*Esr1*), estrogen receptor 2 (*Esr2*), and *Kiss1* mRNA levels were also measured in both MBH and PoA. Quantitative PCR analysis was carried out in the MBH and the PoA. After decapitation, the PoA and the MBH were rapidly dissected. The brain was placed ventral side up. The dissection began by two sagittal incisions along the lateral hypothalamic sulci. Two transversal incisions of 2 mm were made 2 mm ahead from the anterior boundaries of the optic chiasm and along the caudal margin of the mammillary bodies. Finally, a frontal incision was made 2 mm under the ventral surface of the hypothalamus.

Total RNA was extracted from the MBH, medial PoA, and total ovarian tissue using a Universal RNA mini kit (Qiagen, Venlo, Netherlands). Prior to extraction, ovarian tissue was homogenized using a Mikro-Dismembrator S (Sartorius Stedim Biotech, Göttingen, Germany). Five hundred nanograms of RNA for each sample was reverse transcribed using the Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany). For real-time quantitative PCR reactions, the cDNA of our samples was diluted 10-fold and 4 μL was added to a mix of 5 μL of FastStart Universal SYBR Green Master mix (Roche Diagnostics), 0.4 μL of nuclease-free water, and 0.3 μL of forward and reverse primers (see primer sequences in Table 1). The samples were run in triplicate using a LightCycler 480 thermocycler (Roche Diagnostics). Ct values were obtained from each individual amplification curve and the average Ct was calculated for each target gene in each sample. Quantification of relative gene expression was performed using an original program developed on Python 2.7.13 according to the ΔΔCt method implemented with the Pfaffl equation, which takes into account reaction efficiency depending on primers (49). All assays had efficiencies between 1.9 and 2.1. β-Actin was used as a housekeeping gene.

Statistical analysis

Data analyzed with a nonparametric test were expressed as median and interquartile range (IQR). Numeric values of data analyzed with a two-way ANOVA were expressed as mean ± SEM. When normality and homogeneity of variance were not accomplished, a two-group comparison Mann–Whitney nonparametric test was carried out. When making multiple comparisons, α was adjusted by using the Bonferroni correction. Effect sizes were calculated using the equation $r = z/\sqrt{N}$, where z is consistent with the adjusted normally distributed variable value. Estrous cyclicity after adult BPA exposure was analyzed using a McNemar test comparing the pre-exposure period vs the exposure period and the exposure period vs the postexposure period using each group as its own control. When the conditions for this test were not fulfilled, a mid-*P* McNemar test based on the binomial test was carried out. Estrous cyclicity after neonatal exposure and LH surge data after adult exposure were analyzed by using a repeated-measures ANOVA followed by the Tukey test for multiple comparisons and η^2 and Cohen d as an indicator of effect size. The level of statistical significance was a P value <0.05. Data were analyzed using Prism 6.01 (SCR_002798, GraphPad Software).

Table 1. Primer Sequence

Gene		Sequence	Annealing Temperature, TM	Length, bp	Accession Number
β-Actin	F	5′−*C GCGAGTACAACCTTCTTGC*−3′	59.6	200	NM_031144.3
	R	5′–*ATACCCACCATCACACCCTG*–3′	59.1		
Kiss1	F	5′–*GGAGCCACTGGCAAAAATGG*–3′	57.3	86	NM_181692.1
	R	5′–*GCCAGGCATTAACGAGTTCC*–3′	56.4		
Esr1	F	5′–*GCCGAGGTACAGATTGGCTT*–3′	57.4	188	NM_012689.1
	R	5′–*TGCTCATCGTCTTCATGCCC*–3′	57.6		
Esr2	F	5′–*TGAGGCGGACAGACTACAGA*–3′	57.4	147	NM_012754.1
	R	5′–*TATGAGGAACACCGCCACAC*–3′	57.3		
Gat2	F	5′–*TTCATCGGGCTCATTATGCTCA*–3′	59.9	193	NM_133623.1
	R	5′–*TGATAAGAGGCCACGGCTTG*–3′	60.1		
Gad2	F	5′–*GCACCTGTGACCAAAAACCC*–3′	59.9	73	NM_012563.1
	R	5′–*AGGTCTGTTGCGTGGAGAAG*–3′	60.0		
Per1	F	5′–*CTCTCCGCAACCAGGATACC*–3′	59.9	73	NM_012563.1
	R	5′–*ACATCCTGGGGAGCTAGGAG*–3′	60.0		
Per2	F	5′–*CGAAGCGCCTCATTCCAGA*–3′	59.9	73	NM_012563.1
	R	5′–*TGCTCATGTCCACGTCTTCC*–3′	60.0		
Bmal1	F	5′–*AATGCGATGTCCCGGAAGTT*–3′	59.9	73	NM_012563.1
	R	5′–*TCTGTGTATGGGTTGGTGGC*–3′	60.0		
Clock	F	5′–*CAGTCTCAGACCCTTCCTCAAC*–3′	59.9	73	NM_012563.1
	R	5′–*TTGAGACATCGCTGGCTGTG*–3′	60.0		
Pnx	F	5′–*GCGCTCATATTCGGAGGCTT*–3′	59.9	73	NM_012563.1
	R	5′–*GCGCTCATATTCGGAGGCTT*–3′	60.0		

Quantitative RT-PCR primers, annealing temperature and PCR product size used in quantitative PCR. Primers were at a concentration of 10 μM, and experiments were run at 45 cycles and 60°C as T_m.

Abbreviations: F, forward; R, reverse.

Results

Persistent or transient estrous cycle disruption after neonatal or adult BPA exposure

As we had previously shown (15), pubertal onset was affected by neonatal exposure to BPA with opposing effects depending on the dose. Vaginal opening occurred 3 days later on average after neonatal exposure to BPA at 25 ng/kg/d and 3 days earlier after BPA at 5 mg/kg/d. The time period from the day of vaginal opening to the first full regular cycle was significantly increased (5.5 and 6.1 days, respectively) after exposure to both the low BPA dose ($U = 7$, $z = 2.0$, $P < 0.05$) and the high BPA dose ($U = 6.5$, $z = 2.2$, $P < 0.05$, $r = 0.8$) [Fig. 1(a), left]. Subsequently, neonatal exposure to both the low and high dose of BPA significantly decreased the percentage of cycling females [Fig. 1(a), right; see (50)]. At PND 90 to 105, whereas the control group showed regular cycles

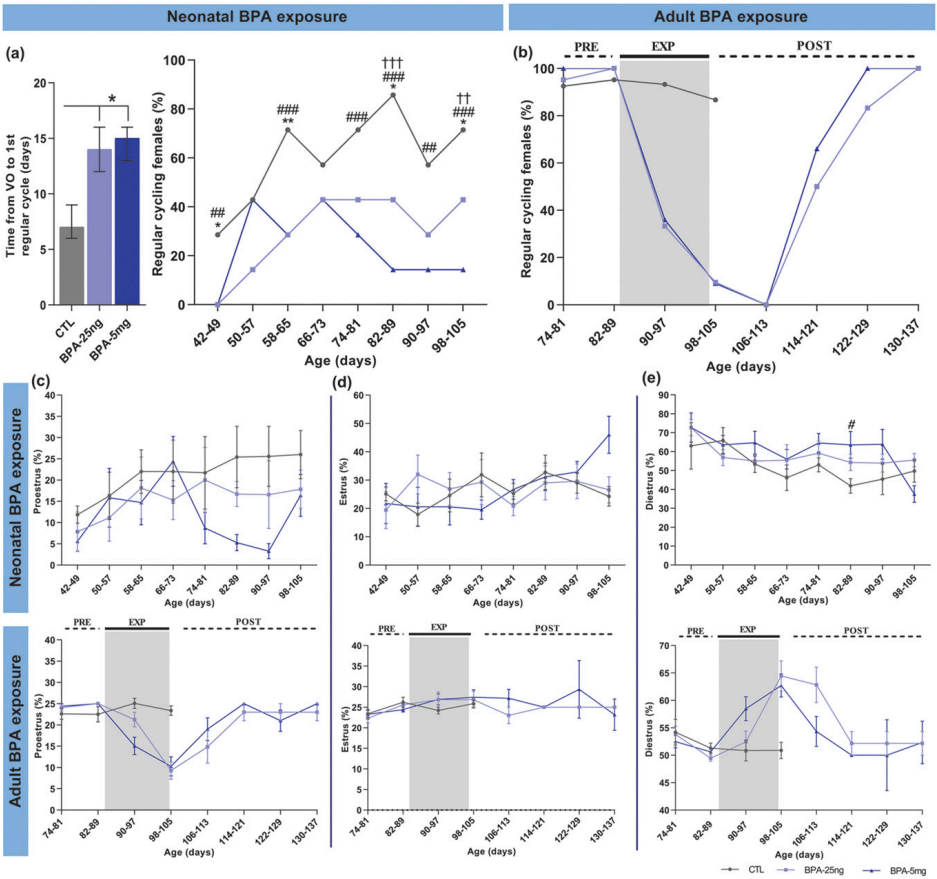

Figure 1. Characteristics of estrous cycle after neonatal (PND 1 to 15) and adult (PND 90 to 105) exposure to BPA (25 ng/kg/d or 5 mg/kg/d) or corn oil (control). (a) Left: Average time from day of vaginal opening (VO) to first regular cycle. Right: Percentage of females exhibiting regular cycling from 42 to 105 d of age after neonatal BPA exposure (n = 7 per group). (b) Percentage of females exhibiting regular cycling 2 wk before, during (control, n = 15; BPA at 25 ng, n = 22; BPA at 5 mg, n = 21), and 4 wk after adult BPA exposure (BPA at 25 ng, n=11; BPA at 5 mg, n = 11). (c–e) Percentage of time spent in (c) proestrus, (d) estrus, and (e) diestrus after neonatal (top) or adult (bottom) exposure to BPA. Neonatal BPA exposure data were analyzed using a repeated-measures ANOVA (Tukey test for multiple comparisons); adult exposure comparisons were analyzed using the mid-*P* McNemar test. Data are expressed as (a) mean (IQR) and (c–e) percentage ± SEM. Control vs BPA at 25 ng: $^{*}P < 0.05$; $^{**}P < 0.01$. Control vs BPA at 5 mg: $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$. BPA at 25 ng vs BPA at 5 mg: $^{\dagger\dagger}P < 0.01$; $^{\dagger\dagger\dagger}P < 0.001$. CTL, control; EXP, exposure; POST, postexposure; PRE, pre-exposure.

in 71% of the females, the BPA-exposed groups showed only 43% (BPA at 25 ng) and 14% (BPA at 5 mg) of regularly cycling females. Although the BPA-treated groups showed a trend toward less time spent in proestrus and more time in diestrus, most values were not significantly different except on PND 82 to 89 when comparing control and the high BPA dose [Fig. 1(c)–1(e); see (50)].

We sought to compare the effects of neonatal exposure to BPA on estrous cycles obtained above with effects observed during and after adult exposure. Adult exposure to BPA for 15 days caused significant alterations of the estrous cycle [Fig. 1(b); Table 2]. During exposure to 25 ng/kg/d or 5 mg/kg/d of BPA, the proportion of females with regular cycle decreased markedly and similarly to 12% and 9%, respectively ($\chi^2 = 13.1$ and 12.1, $P < 0.001$). This effect persisted for 1 week after the end of BPA exposure. Subsequently, the percentage of regularly cycling females was restored to 100% 4 weeks after the end of exposure to the two doses of BPA, indicating the reversibility of the effect. The alteration in cyclicity was characterized by a significant decrease of the time spent in proestrus (BPA at 25 ng, $\chi^2 = 12.5$, $P < 0.001$; BPA at 5 mg, $\chi^2 = 16.1$, $P < 0.001$) as well as an increase of the time spent in diestrus (BPA at 25 ng, $\chi^2 = 8.5$, $P < 0.01$; BPA at 5 mg, $\chi^2 = 4.92$, $P < 0.05$) [Fig. 1(c)–1(e)]. Time spent in estrus was not affected by exposure to BPA. Time spent in proestrus was restored to 23% to 25% after the end of exposure to both doses, but exposure vs postexposure comparisons by using the mid-*P* McNemar test did not reach significance. Ninety-two percent of the control females showed regular cycles during the pre-exposure and the exposure periods.

Neonatal or adult BPA exposure alters early or late stages of folliculogenesis, respectively

The number of primordial follicles in the ovaries evaluated at PND 105 in the control group was significantly decreased after neonatal exposure to both the low dose of BPA ($U = 6.0$, $z = 3.4$, $P < 0.001$, $r = 1.5$) and the high dose of BPA ($U = 9.0$, $z = 2\ 0.7$, $P < 0.001$, $r = 1.2$) [Fig. 2(a)]. Moreover, the number of atretic follicles per ovary was increased after neonatal exposure to the high dose of BPA ($U = 12.0$, $z = -2.4$, $P < 0.05$, $r = 1.1$). The low dose of BPA did not affect the number of atretic follicles. Cystic follicles were absent in the control ovaries and present after neonatal exposure to both doses of BPA. Ovarian weight was significantly reduced after neonatal exposure to the low BPA dose ($U = 6.5$, $z = 2.2$, $P < 0.05$, $r = 0.8$). Although a similar average weight reduction was observed after exposure to the high BPA dose, the difference was not found to be significant [Fig. 2(b)].

The number of antral follicles was significantly decreased 24 hours after the last day of adult exposure to the low dose ($U = 0.0$, $z = 2.5$, $P < 0.05$, $r = 1.1$) or high dose of BPA ($U = 0.0$, $z = 2.3$, $P < 0.05$, $r = 1.0$) [Fig. 2(c)]. The number of corpora lutea was also decreased after exposure to BPA, but only significantly for the high BPA dose ($U = 0.0$, $z = 2.5$, $P < 0.05$, $r = 1.1$).

Table 2. Estrous Cyclicity After Adult Exposure to BPA

		PRE vs BPA						BPA vs POST					
	Group	**n**	**=**	**<**	**>**	***z***	***P***	***n***	**=**	**<**	**>**	***z***	***P***
Regular cycling	Corn oil	15	8	3	4	0.57	–	–	–	–	–	–	–
females	BPA-25ng	22	7	15	0	13.06	[a]	6	1	5	0	7.2	[b]
	BPA-5mg	21	7	14	0	12.07	[a]	6	1	5	0	7.2	[b]
Proestrus	Corn oil	15	8	2	5	2.29	–	–	–	–	–	–	–
	BPA-25ng	22	4	17	1	12.50	[a]	6	2	4	0	6.25	–
	BPA-5mg	21	3	18	0	16.06	[a]	6	3	3	0	5.33	–
Estrus	Corn oil	15	12	1	2	1.33	–	–	–	–	–	–	–
	BPA-25ng	22	17	1	4	3.20	–	6	6	0	0	–	–
	BPA-5mg	21	16	2	3	0.80	–	6	4	2	0	0.5	–
Diestrus	Corn oil	15	9	2	4	1.50	–	–	–	–	–	–	–
	BPA-25ng	22	5	15	2	8.47	[b]	6	3	3	0	5.33	–
	BPA-5mg	21	8	11	2	4.92	[c]	6	2	4	0	2.25	–

The number of regular cycles and the number of days spent in proestrus, estrus, and diestrus were quantified in each of three periods: pre-exposure (PRE), exposure (BPA), and postexposure (POST). A McNemar test was carried out for comparing treatment versus pretreatment and a mid-*P* McNemar test based on the binomial test was used for comparing treatment versus posttreatment. Data are expressed as number of animals in each group.

Abbreviations: =, number of females that did not show differences between any of both compared periods; <, number of females that showed a decrease in regular cyclicity; >, number of females that showed an increase in regular cyclicity; *z* = McNemar or mid-*P* McNemar statistical value.

[a] $P < 0.001$.

[b] $P < 0.01$.

[c] $P < 0.05$.

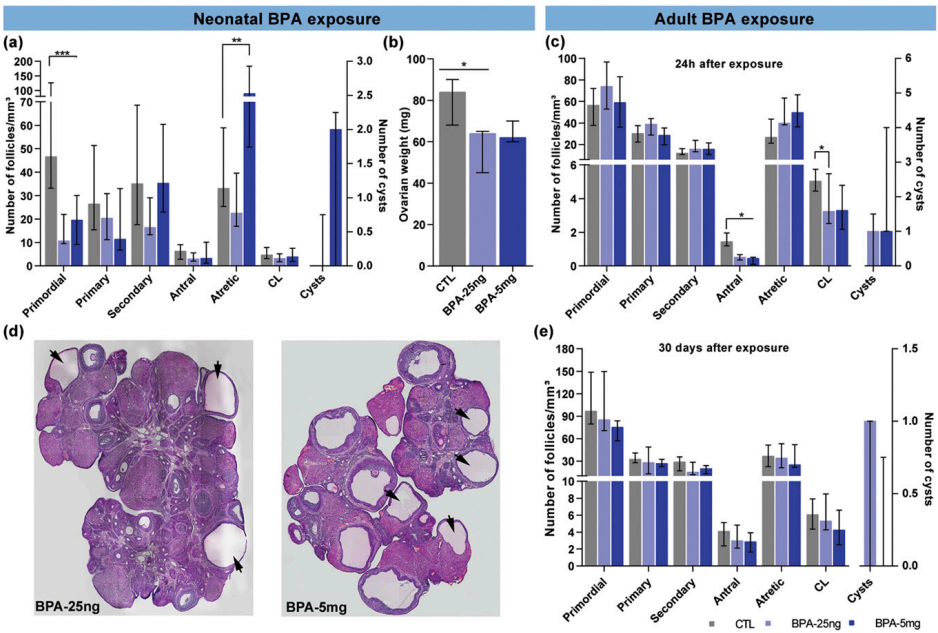

Figure 2. Effects of neonatal (PND 1 to 15) and adult (PND 90 to 105) exposure to BPA (25 ng/kg/d or 5 mg/kg/d) or corn oil (control) on ovarian weight and folliculogenesis during diestrus. Quantification of (a) follicles and corpora lutea and (b) ovarian weight after neonatal BPA exposure (n = 7 per group). Quantification of follicles and corpora lutea (c) 24 h or (e) 30 d after adult BPA exposure (n = 6 per group). (d) Representative ovarian sections obtained from animals 24 h after adult exposure using the slide scanner DotSlide with a magnification of ×10. Arrows depict the presence of some cystic follicles. Follicles were quantified in every other section and normalized by ovarian volume (mm^3). The data were analyzed using a Mann–Whitney test and represented as median and IQR. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control. CTL, control.

The number of atretic follicles tended to increase, although not significantly in the animals exposed to the two doses of BPA compared with controls. Cystic follicles were found in the ovaries of females exposed to the low and the high doses of BPA but were never observed in control animals [Fig. 2(d)]. The effect of BPA on ovarian histology was reversible, because 30 days after BPA exposure, the number of follicles per volume at the different stages of folliculogenesis was no longer significantly different among the control and exposed animals [Fig. 2(e)]. However, the animals previously exposed to the low and high BPA dose still showed cystic follicles, which were not observed in the control group [Fig. 2(e)].

Adult BPA exposure transiently disrupts the LH surge

Basal serum and pituitary levels of LH and FSH measured during diestrus, 24 hours after the last day of adult exposure [Fig. 3(a)–3(d)], were not significantly affected. Because the proestrus timing appeared to be disrupted by BPA exposure, we characterized the spontaneous LH surge before, during, and after a 15-day exposure to BPA. LH secretion was measured during two consecutive afternoons 48 hours after estrus to identify the spontaneous LH surge. The LH surge was significantly delayed during the exposure to both doses of BPA [see representative profile in Fig. 3(e)]. A systematic delay of 2.6 and 2.8 hours on average was observed during exposure to the BPA at 25 ng group ($P < 0.05$) and BPA at 5 mg group ($P < 0.01$), respectively [Fig. 3(f)]. The timing of the LH surge was restored 1 month after the end of exposure. Additionally, the high BPA dose significantly blunted the LH surge during the second week of exposure compared with the pre-exposure period ($P < 0.01$) [Fig. 3(g)]. The effect was reversible, as the LH surge amplitude was restored after exposure. The amplitude of the LH surge was not significantly affected by the low BPA dose.

We have shown previously that neonatal exposure to BPA disrupts GnRH pulsatile secretion (15). In the current study, we studied the effects of adult exposure to BPA on GnRH pulsatile secretion. Hypothalamic

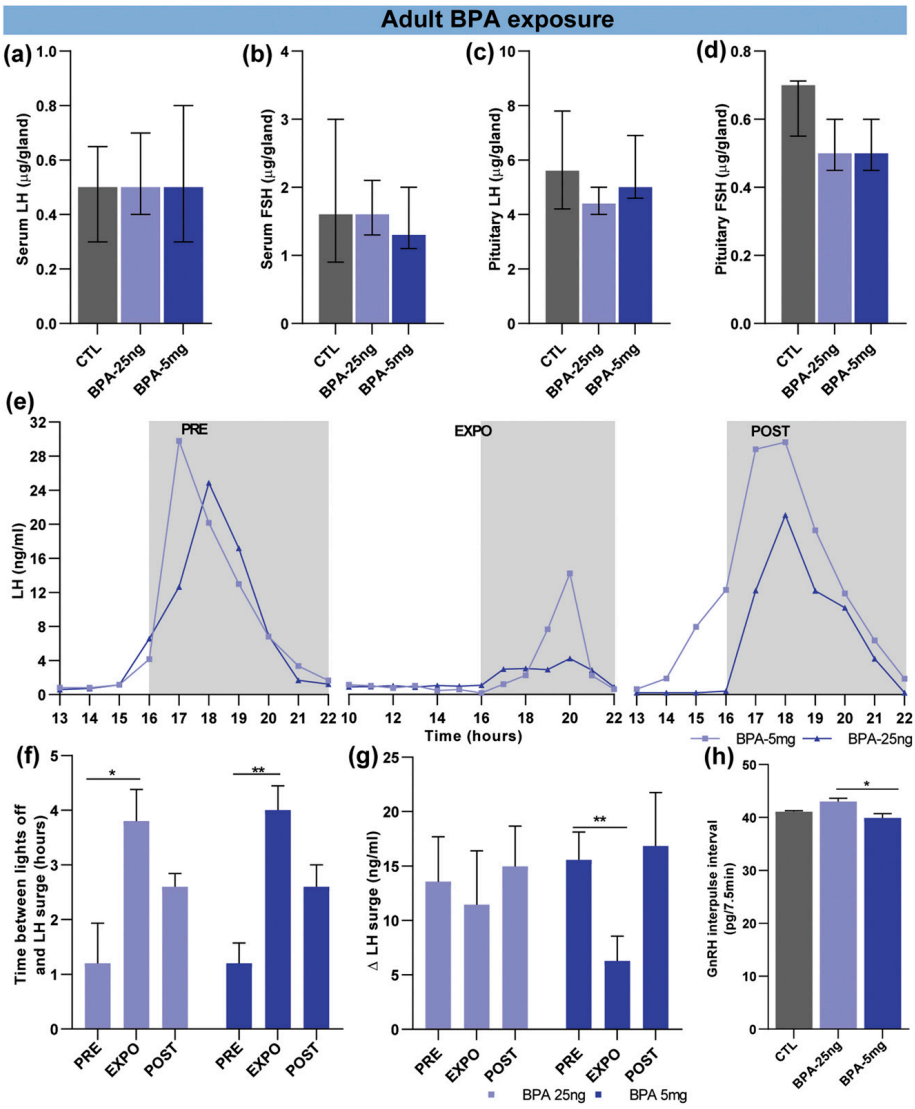

Figure 3. Effects of adult (PND 90 to 105) exposure to BPA (25 ng/kg/d or 5 mg/kg/d) or corn oil (control) on serum and pituitary LH and FSH, GnRH pulsatile secretion, and the preovulatory LH surge. (a and b) Serum and (c and d) pituitary LH (diluted 2000×) and FSH (diluted 500×) levels. Pituitary LH and FSH were multiplied by their dilution factor to obtain an amount of micrograms per gland (control, n = 9; BPA at 25 ng, n = 12; BPA at 5 mg, n = 7). Samples were collected 24 h after the last BPA or corn oil administration during diestrus stage. (e) Representative LH surge from two females exposed to either the low or high BPA dose. The gray area represents the dark phase. (f) LH surge timing after beginning of the dark phase (4:00 PM). (g) LH surge amplitude before (PRE), during (EXPO), and after (POST) adult BPA exposure (BPA at 25 ng, n = 5; BPA at 5 mg, n = 5). (h) GnRH interpulse interval *in vitro* using hypothalamic explants obtained on PND 106, that is, 24 h after the last administration of BPA or corn oil (control) in adult female rats (n = 4 per group). Data were analyzed using a two-way ANOVA and represented as mean ± SEM. $*P < 0.05$, $**P < 0.01$ vs control.

explants obtained from females in diestrus 24 hours after the end of exposure to BPA were incubated individually to study pulsatile GnRH secretion [Fig. 3(h)]. The GnRH interpulse interval was not significantly different between the control group (41.1 ± 0.2 minutes) and BPA-exposed females, contrary to what was observed after neonatal exposure (15). The GnRH interpulse interval was, however, significantly longer after exposure to the low dose when compared with the high dose of BPA ($U = 0.0, z = 2.2, P < 0.05, r = 0.5$), with an average difference of 3.1 minutes.

We carried out quantitative PCR analysis of the estrogen receptors *Esr1* and *Esr2*, *Kiss1*, the clock genes *Per1*, *Per2*, and *Clock*, and the peptide *Pnx* in the MBH [Fig. 4(a)] and PoA (Fig. 4) of adult control and BPA-exposed females. Genes involved in GABAergic transmission were studied as well because they had been previously shown to be sensitive to neonatal exposure to a low and high dose of BPA (15). MBH and POA were dissected in females in diestrus 24 hours after the last day of exposure. In the MBH, *Per1* was found to be significantly decreased after exposure to the high ($U = 0.0, z = 3.2, P < 0.05, r = 1.2$) and the low BPA dose ($U = 2.5, z = 3.0, P < 0.05, r = 1.2$). *Pnx* was significantly increased after exposure to the high BPA dose and decreased after exposure to the low BPA dose both in the PoA and MBH. Relative mRNA expression of *Esr1*, *Esr2*, *Kiss1*, *Gad2*, and *Gat2* was not significantly affected by BPA in the MBH or the PoA.

Discussion

In the current study, to our knowledge, we provide the first evidence that adult exposure to a low environmentally relevant dose of BPA, in the range of nanograms, disrupts the preovulatory LH surge and leads to abnormal estrous cycle and folliculogenesis. Such disruption is reversible after adult exposure to BPA whereas it persists into adulthood following neonatal exposure, indicating a disruption of ovarian programming.

Few studies have directly compared the windows of sensitivity to BPA. Nikaido *et al.* (22, 23) have used BPA

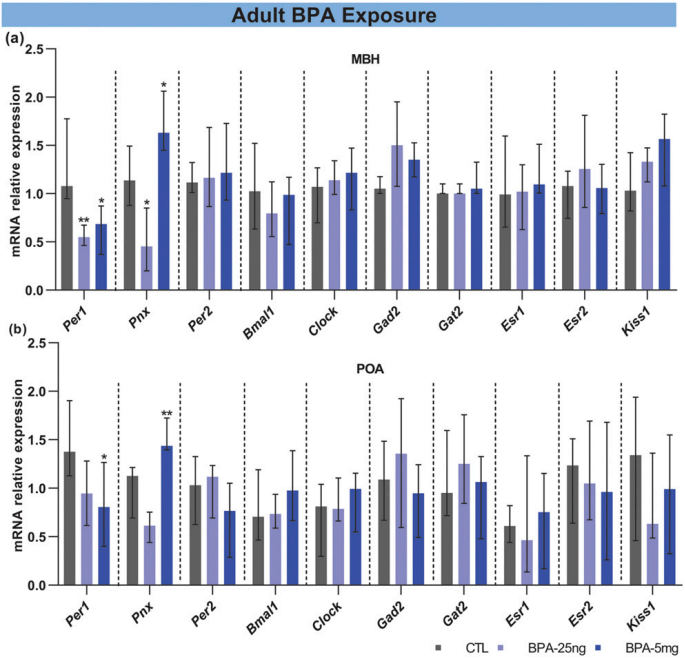

Figure 4. Effects of adult (P90 to 105) exposure to BPA (25 ng/kg/d or 5 mg/kg/d) or corn oil (control) on relative gene expression in the (a) MBH or (b) PoA. Females in diestrus 24 h after the last BPA dose were analyzed for relative mRNA expression of *Per1*, *Pnx*, *Per2*, *Bmal1*, *Clock*, *Gad2*, *Gat2*, *Esr1*, *Esr2*, and *Kiss1* (control, n = 5; BPA at 25 ng, n = 6; BPA at 5 mg, n = 5). Data were analyzed using a Mann–Whitney test and represented as median and IQR. $*P < 0.05$, $**P < 0.01$ vs control.

during and after the organizational period of sex steroids for reproduction in female mice. CD-1 female mice were exposed to 10 mg/kg/d of BPA for 4 days either prenatally during the last week of gestation or prepubertally starting at PND 15. In both conditions, a reduced presence of corpora lutea was observed at 4 weeks of age, by the time of vaginal opening. This effect had disappeared at 8 and 24 weeks of age. These data indicate some reversibility of BPA effects on luteinization following exposure during and after the organizational fetal window. Although we did not study ovarian histology close after the time of vaginal opening, we report in the current study that the time from vaginal opening to the first complete estrous cycle is markedly increased after neonatal exposure to BPA. This is consistent with a reduced likelihood of ovulation and corpora lutea formation. Also, the presence of corpora lutea by 18 weeks of age is not reduced after neonatal exposure in the current study, in agreement with Nikaido *et al.*'s findings. The importance of the selected endpoints is emphasized by our data because the reduced representation of primordial follicles appears here to be the major expression of disrupted ovarian organization.

Although the alterations of estrous cyclicity persist after neonatal exposure, they appear to be transient during adult exposure to BPA. During 2 weeks of adult exposure to a very low or high dose of BPA, altered estrous cyclicity occurs together with disruption of the late stages of folliculogenesis (antral follicles and corpora lutea). Importantly, all of these effects appear to have disappeared 1 month after stopping the exposure to BPA.

Both neonatal and adult exposure lead to alterations characterized by a decrease in the percentage of time spent in proestrus and an increase in the time spent in diestrus. Wang *et al.* (51) reported that proestrus was reduced and diestrus increased in 3-month-old mice after fetal exposure to a relatively low dose of BPA (500 ng/kg), in agreement with our findings after neonatal exposure of rats to 25 ng/kg. In our study, as the time spent in estrus was not affected by BPA, we hypothesized that the timing of ovulation during proestrus could be affected. To verify this hypothesis, we measured the LH surge using serial blood sampling on the day of the expected proestrus, and our results showed a systematic delay of the LH surge caused by both BPA doses together with a decrease in LH surge amplitude. Gestational exposures to much higher concentrations of BPA in sheep have been previously shown to lead to a dampened (52) and slightly delayed LH surge (53). Other studies have reported decreased amplitude of the LH surge after gestational or prepubertal exposure to *p-tert*-octylphenol (54), perfluorooctanesulfonic acid (55), atrazine (56) or polychlorobiphenyls (57). However, to our knowledge, our study is the first one showing such a systematic delay during adult exposure to a low dose of BPA.

Prenatal exposure to BPA has been shown to modify hypothalamic gene expression and behavior in mice and rats (58, 59), supporting central mechanisms for BPA effects. We also found that GABA neurotransmission was involved in the neonatal effects of BPA on the neuroendocrine control of GnRH secretion (15). In our previous studies using a model of pulsatile GnRH secretion by hypothalamic explants *ex vivo*, we have shown that neonatal exposure to BPA results in opposing effects on the GnRH interpulse interval depending on the dose (15). In the current study, adult exposure also leads to some opposing central effects of BPA on the GnRH interpulse interval during diestrus studied *ex vivo*. Whereas a low BPA dose slightly increases the GnRH interpulse interval, the high BPA dose results in a decrease of the interpulse interval. Although adult exposure to BPA results in the same pattern of change in GnRH secretion than does neonatal exposure, the effect is quantitatively less important. However, both doses significantly disrupted the LH surge, which brings more evidence regarding the neuroendocrine disruption of ovulation caused by BPA. Additionally, Veiga-Lopez *et al.* (53) have shown that the preovulatory estradiol rise in prenatal BPA-treated female sheep was similar to that of controls, which indicates that the ovarian signal is normal and the defect involves the neuroendocrine control of the LH surge generation. We have shown in the current study that the expression of phoenixin (*Pnx*), a newly discovered hypothalamic peptide linked to reproduction, is sensitive to adult exposure to BPA. *Pnx* is thought to be involved in the preovulatory LH surge through stimulation of GnRH and kisspeptin release and has been recently shown to be sensitive to BPA (60, 61). Knockdown of PNX receptor (GPR173) using intracerebroventricular injection of small interfering RNA doubled the length of the estrous cycle in female rats and eliminated the *Pnx*-induced increase in plasma LH (62, 63). Therefore, the decrease in *Pnx* expression that we observed indicates that this crucial regulator of GnRH and kisspeptin could be involved in the disruption of ovulation caused by BPA. Conversely, the high BPA dose led to an increase in *Pnx* expression in the MBH and POA. Such dose-related opposite effects on hypothalamic genes have been previously reported by our team after neonatal exposure to BPA (15). Because higher concentrations of BPA have been shown to decrease GPR173 expression in immortalized hypothalamic neurons, the increased expression of *Pnx* after the high BPA dose could be interpreted as reactional.

The anteroventral periventricular nucleus (AVPV) is a region critical for the occurrence of the LH surge and is known to be sensitive to endocrine disruptors. Decreased *Kiss1* and *Esr1* mRNA expression in the AVPV was observed on postnatal day 10 after exposure to BPA (64) although adult exposure might increase *Kiss1* mRNA expression in the AVPV (65). Altogether, a decreased hypothalamic–pituitary sensitivity, caused by a failure of the AVPV to respond to peripheral signals, could explain the impaired LH surge caused by BPA. However, mRNA levels of estrogen receptors and Kisspeptin were not affected either in the mPoA or the MBH in our model. Further studies should look at specific AVPV expression. The occurrence of the preovulatory LH surge depends on the master circadian clock within the suprachiasmatic nucleus together with rising ovarian estrogen levels. The clock genes *Per1* and *Bmal1* in the AVPV play a critical role as integrator of ovarian and circadian signals to time the LH surge (66, 67) and appear to be sensitive to endocrine disruption (68). Recently, Loganathan *et al.* (68) showed that BPA was able to alter *Bmal1* and *Per2* expression in immortalized hypothalamic neurons. Our data indicate that *Per1* expression in the hypothalamus is sensitive to adult exposure to BPA and suggest that clock genes could be the central link explaining the effect of BPA on LH surge timing. Further studies will need to look at *Per1* expression in the AVPV throughout the afternoon of the proestrus.

Alternatively, disruption of the LH surge by BPA could be explained through an indirect effect on energy balance and homeostasis. Indeed, neuropeptide Y (NPY) and POMC are known to be involved in the regulation of the LH surge in rats (60–71) and to be sensitive to exposure to BPA. Gestational exposure increases NPY and AGRP and decreases POMC hypothalamic expression in male rats (72). Female mice exposed *in utero* and during lactation show reduced proopiomelanocortin fiber innervation into the paraventricular nucleus of the hypothalamus and increased adiposity and leptin serum levels (73). Interestingly, recent *in vitro* data using hypothalamic cell lines and primary cultures indicate that BPA requires *Bmal1*, a clock gene known to increase NPY expression in hypothalamic neurons (68).Whether the effects of BPA on the LH surge are mediated by targeted changes in components of the energy balance remains unknown.

The exposure to BPA during gestation or neonatal life can affect ovarian structure and function (74). Fetal exposure to BPA was shown to increase cells in germ cell nests and to reduce primordial follicles (51). The neonatal period is critical for ovarian differentiation because formation of primordial follicles is not completed until PND 3 to 4 and initial recruitment takes place during neonatal life. Thus, disturbances in early stages of folliculogenesis can also occur after neonatal exposure to BPA (75). Our findings indicate that reduced pools of primordial follicles or antral follicles could reflect insults during or after development, respectively. In the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) study (35), exposure to BPA at 2.5 and 250 μg/kg/d from gestational day 6 to PND 21 resulted in reduced primordial, primary, and preantral follicles at PND 21. However, irrespective of stopping exposure to BPA at PND 21 or continuing until the end of experiment, there were no longer any alterations of folliculogenesis at 3 and 6 months of age. By 1 year of age, cystic follicles were found only after exposure to 25 mg/kg/d of BPA until PND 21. These data indicate possible developmental effects of BPA although they appear to be transient and reversible, even during sustained exposure. Among the factors possibly accounting for discrepancies between the CLARITY-BPA study and the present one, the dose of BPA, the route of administration, and the age window of exposure could play some role as well as differences in rat strain (Wistar vs Sprague-Dawley). In some studies, the number of primary follicles was either reduced (29) or increased (76) after early postnatal exposure to BPA in the microgram/kilogram dose range. Interestingly, fetal exposure of mice (from gestational day 6 to birth) to a lower dose of BPA (500 ng/kg) than in the CLARITY-BPA study resulted in the reduced presence of antral follicles at PND 21 (77) and at 3 months of age (34). These findings are consistent with ours regarding persistent effects in adulthood after early life exposure, although those authors did not find any reduction in primordial follicles (34). In similar conditions, however, the number of primordial follicles was reduced on PND 4, indicating that both the age at exposure and the age at evaluation matter (51). Taken together, these data point to the requirement of additional studies involving postnatal and sustained exposure to very low doses of BPA in the nanogram/kilogram range.

Human exposure to BPA is sustained throughout life and provides the rationale for lifelong exposure, as done in the CLARITY-BPA study (78). We elected to expose the animals to BPA transiently for 2 weeks because a transient exposure was required to evaluate whether effects persisting into adulthood could result from neonatal exposure. Likewise, transient exposure in adulthood was necessary to study possible reversibility of the effects after exposure in conditions comparable with those used neonatally. In contrast to the CLARITY-BPA study in rats and other studies in mice, our conditions did not include fetal exposure (34, 51, 77, 79). The subcutaneous route of administration was indispensable for reliable administration of BPA to

neonatal rats, particularly using such a very low dose as discussed previously (15). This required control for contamination by other BPA sources through the use of low-phytoestrogen pellets, glass bottles, and BPA-free cages. The oral route could not allow reliable administration of BPA doses in the range of nanograms/kilograms. This possibly explains that such very low doses were not used in the CLARITY-BPA study (78). Although the oral route is consistent with the human conditions of exposure, oral gavage as done in the CLARITY-BPA study can account for confounding factors such as stress and bypass of oral absorption (80). Comparable serum levels of BPA and uridine 5′-diphosphate-glucuronosyltransferase, the enzyme that conjugates BPA, have been reported after oral and subcutaneous administration neonatally (81). However, in another more extensive study in neonatal mice, the systemic levels of free BPA were found to be threefold to fourfold higher after subcutaneous injection than after oral administration (82). Assuming that the pups in our study would have been exposed to BPA levels fourfold higher than using the oral route, such levels (equivalent to 100 ng/kg orally) would still be consistent with human exposure and 25-fold less than the lowest dose used in the CLARITY-BPA study (78). For consistency and to ensure precision in the low levels administered, we also used the subcutaneous route of administration in the adult females. The reversibility of the effects of the low BPA dose after resumption of control conditions supports the evidence that the effects of the very low dose are unlikely resulting from a contaminant because all the management conditions except BPA (vehicle, food, drink, and cages) were identical in the control and treatment settings. That very low dose is far below the no-observed-adverse-effects level and below the European Food Saftey Authority "safe dose". It represents half the average exposure of the general population (1). Following neonatal or adult exposure to BPA, the ovulatory cycle and folliculogenesis are impaired and the effects are similar using a very low dose of BPA or a high dose in the range of milligrams. Collectively, the current study suggests that the effect of BPA on the ovaries is more dependent on the period of exposure in life than the dose of BPA although only two doses were studied and the effects of intermediate doses warrant further studies.

Conclusions

In conclusion, we show that both adult and neonatal exposure to a very low dose of BPA in the range of nanograms can result in alteration of estrous cyclicity and folliculogenesis. Similar alterations are observed using a high dose of BPA. Neonatal exposure leads to effects occurring after exposure and persisting over the long term, suggesting that BPA is able to reprogram the reproductive axis at early stages, particularly by affecting the early follicular development. In contrast, adult exposure to BPA causes effects to occur transiently during exposure because normal cyclicity and folliculogenesis are restored within 1 month after resuming control conditions. Moreover, estrous cyclicity during adulthood seems to be altered by central mechanisms involving the disruption of the LH surge. Our findings imply that when further evaluating BPA adverse effects on the female reproductive axis, very low doses in the range of average environmental exposure should be used with inclusion of the critical neonatal period and addressing both neuroendocrine and ovarian endpoints.

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Disclosure Summary: The authors have nothing to disclose.

Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Appendix D

Experimental study One

Supplemental Material

Persistent vs Transient Alteration of Folliculogenesis and Estrous Cycle After Neonatal vs Adult Exposure to Bisphenol A

		Cycling Females			Diestrus		
Age		q	*d*	*p*	q	*d*	*p*
PND 42-49	CTL vs. BPA-25ng	4,35	3,07	*	1,59	-1,12	
	CTL vs. BPA-5mg	3,70	2,62	**	1,59	-1,12	
	BPA-5mg vs. BPA-25ng	0,65	-0,46		0,00	0,00	
PND 50-57	CTL vs. BPA-25ng	1,06	0,75		0,37	0,26	
	CTL vs. BPA-5mg	2,75	1,95		1,47	1,04	
	BPA-5mg vs. BPA-25ng	1,69	1,20		1,10	0,78	
PND 58-65	CTL vs. BPA-25ng	5,56	3,93	**	1,85	-1,30	
	CTL vs. BPA-5mg	5,13	3,62	***	0,26	-0,18	
	BPA-5mg vs. BPA-25ng	0,43	-0,30		1,59	1,12	
PND 66-73	CTL vs. BPA-25ng	1,73	1,22		1,61	-1,14	
	CTL vs. BPA-5mg	2,95	2,09		1,52	-1,07	
	BPA-5mg vs. BPA-25ng	1,23	0,87		0,09	0,07	
PND 74-81	CTL vs. BPA-25ng	5,67	4,01		1,89	-1,34	
	CTL vs. BPA-5mg	2,41	1,70	***	1,00	-0,71	
	BPA-5mg vs. BPA-25ng	3,27	-2,31		0,89	0,63	
PND 82-89	CTL vs. BPA-25ng	9,64	6,81	*	3,55	-2,51	
	CTL vs. BPA-5mg	3,38	2,39	***	2,03	-1,44	*
	BPA-5mg vs. BPA-25ng	6,25	-4,42	***	1,52	1,07	
PND 90-97	CTL vs. BPA-25ng	4,91	3,47		3,01	-2,13	
	CTL vs. BPA-5mg	2,19	1,55	**	1,38	-0,97	
	BPA-5mg vs. BPA-25ng	2,72	-1,92		1,64	1,16	
PND 98-105	CTL vs. BPA-25ng	8,26	5,84	*	1,99	1,40	
	CTL vs. BPA-5mg	3,95	2,79	***	0,96	-0,68	
	BPA-5mg vs. BPA-25ng	4,31	-3,05	**	2,94	-2,08	

Table D.1. Estrous cycle was studied from PND 42 to PND 105 in females exposed neonatally (PND 1 – PND 15) to corn oil or BPA (25ng/kg/d or 5mg/kg/d). The number of regular cycles and the number of days spent in proestrus, estrus and diestrus were quantified every day. A repeated-measures two-way ANOVA was carried out using both Group and Time (Weeks) as factors. A Tukey post-hoc comparison was used to determine differences between groups. Only measurements with significant differences are shown. Cohen's d (d) was used as indicator of effect size. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n= 7/group.

Appendix E

Experimental study two

Supplemental Material

Multi- and transgenerational disruption of maternal behavior and female puberty by EDC mixture exposure

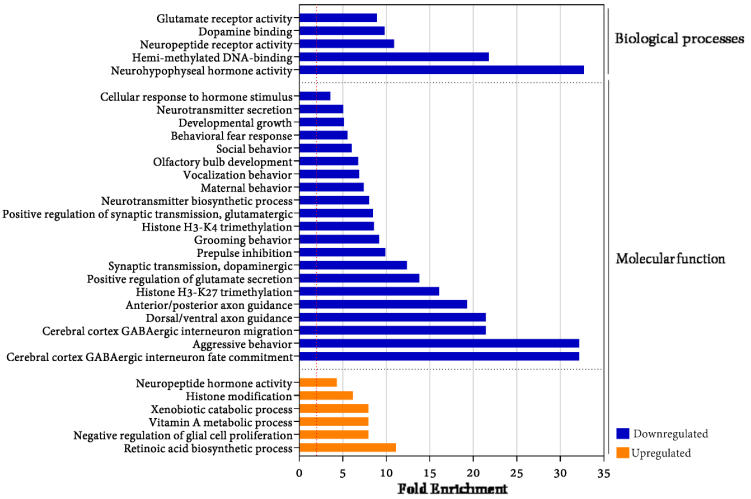

Figure E.1. Fold enrichment of gene ontology (GO) annotations using DAVID pathway analysis across compared groups (CTL vs EDC) in the MBH of F3 generation females at P21 . The gene enrichment analysis grouped the differentially expressed genes using GO annotations data. We selected enriched GO annotations using 2-fold enrichment criteria as a threshold and identifying annotations that were involved in brain and behavioral processes. Those annotations were then categorized in upregulated (orange) or downregulated (blue) annotations.

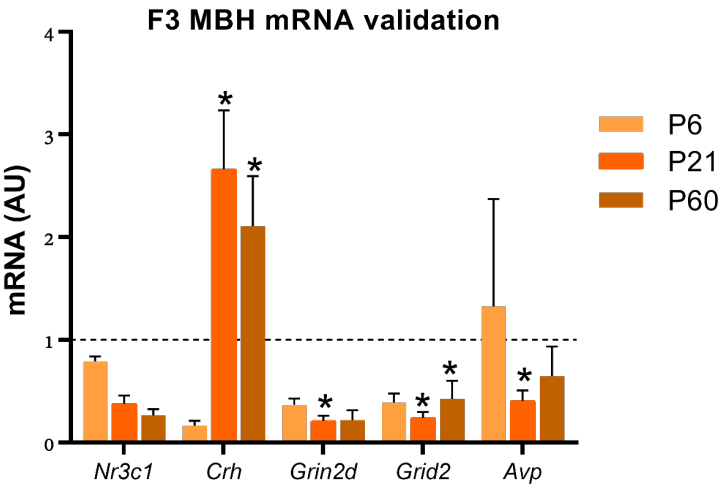

Figure E.2. *Nr3c1, Crh, Grin2d, Grid2* and *Avp* mRNA expression in the female rat ancestrally (F3 generation) exposed to an EDC mixture or vehicle in the MBH of infant (P6), prepubertal (P1) and adult (P60) female rats as determined by qPCR (n=6/group). AU = arbitrary units. RNA expression data were normalized by dividing each individual value by the average of the control group at every time point. Bars represent mean ± s.e.m. (*P < 0.05, **P < 0.01, ***P < 0.001 vs. CTL, Student's t-test).

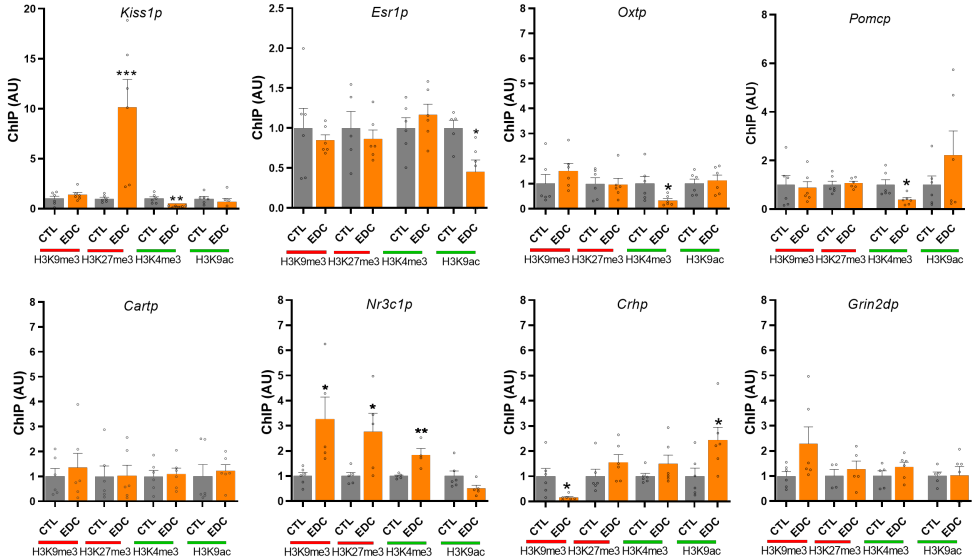

Figure E.3. Abundance of the TrxG-dependent activating marks H3K4me3 and H3K9ac and the PcG-dependent repressive mark H3K27me3 and H3K9me3 at the*Kiss1, Esr1, Oxt, Pomc, Cart, Nr3c1, Crh* and *Grin2d* promoter in the prepubertal MBH of females EDC and control from the F3 generation, as measured by ChIP (n=6/group). Bars represent mean ± s.e.m. (*P < 0.05, **P < 0.01, ***P < 0.001 vs. CTL, Student's t-test).

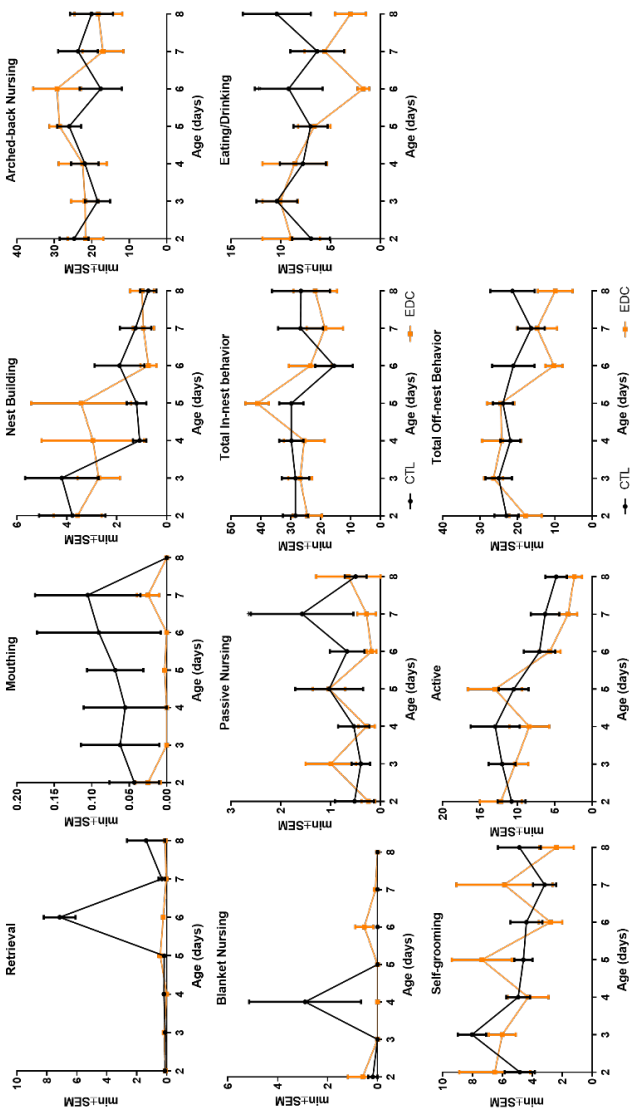

Figure E.4. Maternal behavior displayed by female rats directly exposed to a mixture of EDCs from the F0 generation. Data shows time spent by dams displaying in-nest behavior (retrieval, mouthing, nest building, Arched-back, blancked and passive nursing; and total time spent in-nest) or off-nest behaviors (eating/drinking, self-grooming, being active and total time off-nest) from P2 to P8. Plotted lines represent average of time ± s.e.m.

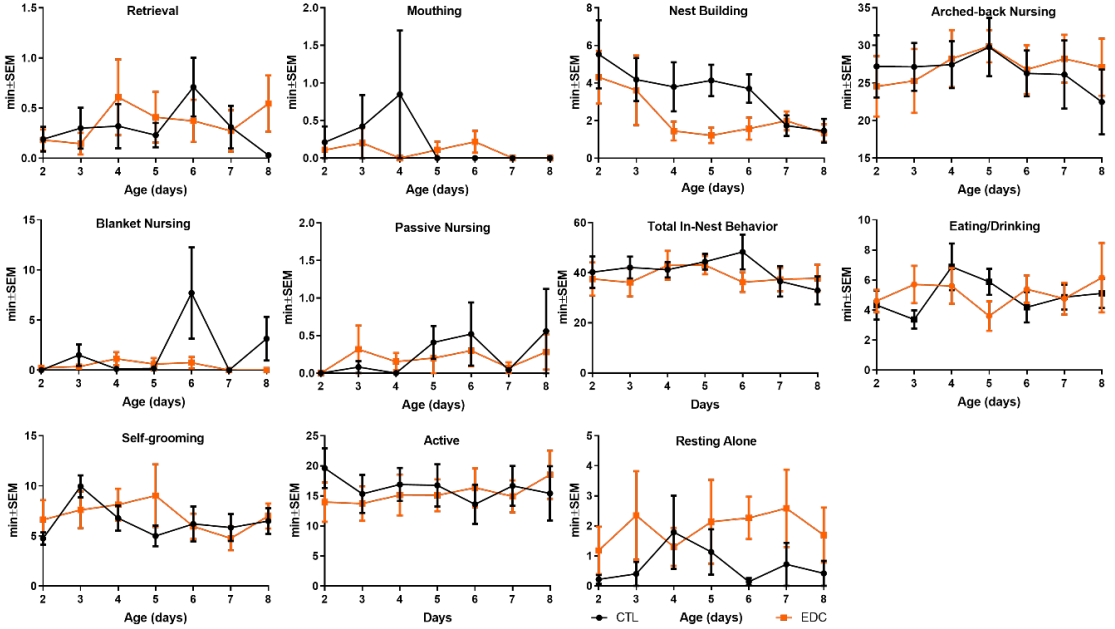

Figure E.5. Maternal behavior displayed by female rats directly exposed to a mixture of EDCs from the F1 generation. Data shows time spent by dams displaying in-nest behavior (retrieval, mouthing, nest building, Arched-back, blancked and passive nursing; and total time spent in-nest) or off-nest behaviors (eating/drinking, self-grooming, being active and total time off-nest) from P2 to P8. Plotted lines represent average of time ± s.e.m.

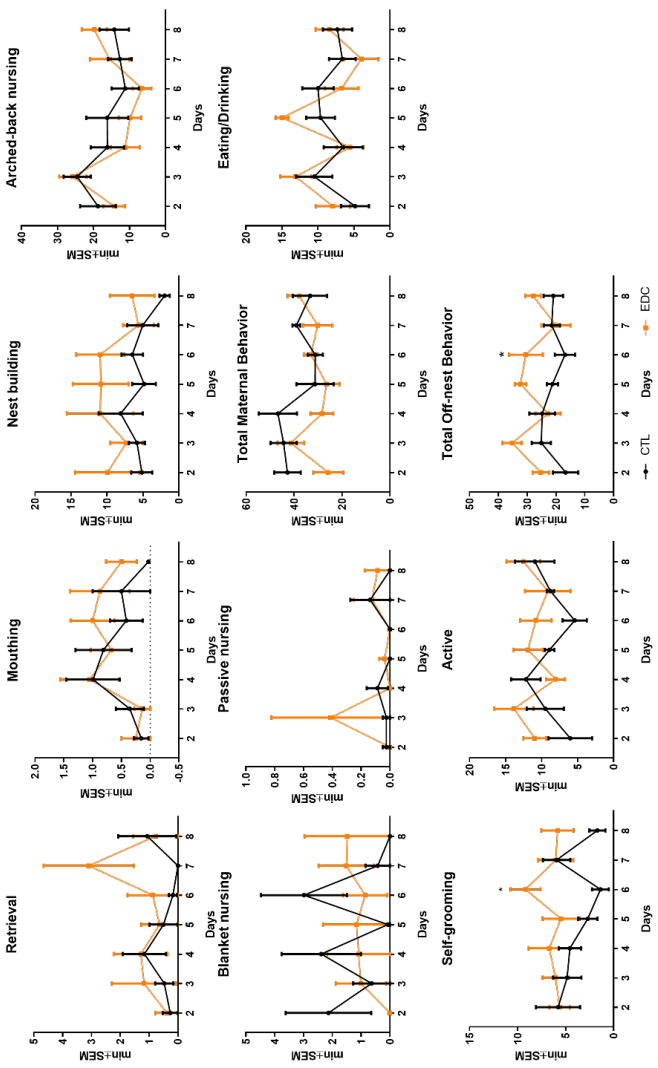

Figure E.6. Maternal behavior displayed by female rats directly exposed to a mixture of EDCs from the F2 generation. Data shows time spent by dams displaying in-nest behavior (retrieval, mouthing, nest building, Arched-back, blancked and passive nursing; and total time spent in-nest) or off-nest behaviors (eating/drinking, self-grooming, being active and total time off-nest) from P2 to P8. Plotted lines represent average of time ± s.e.m.

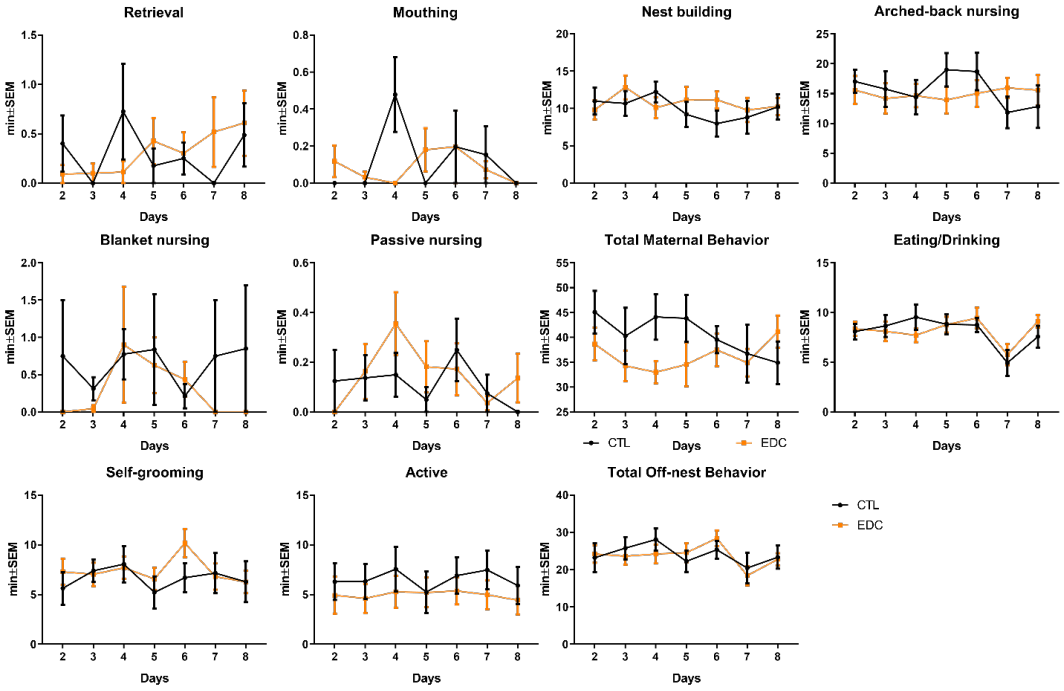

Figure E.7. Maternal behavior displayed by female rats directly exposed to a mixture of EDCs from the F3 generation. Data shows time spent by dams displaying in-nest behavior (retrieval, mouthing, nest building, Arched-back, blancked and passive nursing; and total time spent in-nest) or off-nest behaviors (eating/drinking, self-grooming, being active and total time off-nest) from P2 to P8. Plotted lines represent average of time ± s.e.m.

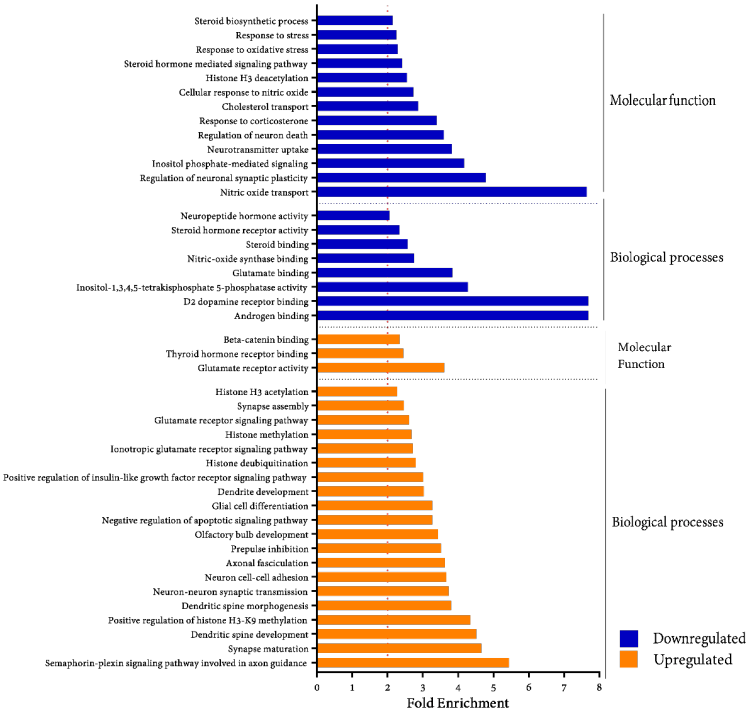

Figure E.8. Fold enrichment of gene ontology (GO) annotations using DAVID pathway analysis across compared groups (CTL vs EDC) in the MBH of F1 generation females at P21. The gene enrichment analysis grouped the differentially expressed genes using GO annotations data. We selected enriched GO annotations using 2-fold enrichment criteria as a threshold and identifying annotations that were involved in brain and behavioral processes. Those annotations were then categorized in upregulated (orange) or downregulated (blue) annotations.

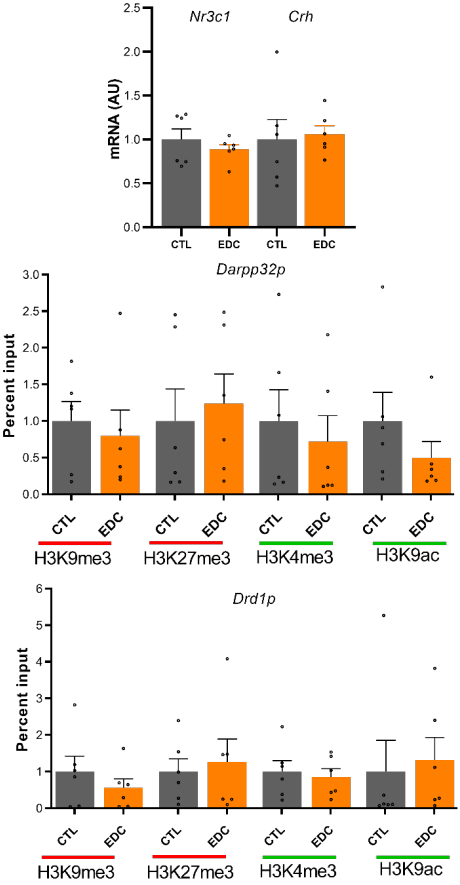

Figure E.9. *Nr3c1* and *Crh* mRNA expression and *Darpp32* and *Drd1* promoter chromatin state in the female rat *in utero* and lactationally (F1 generation) exposed to an EDC mixture or vehicle. (a) Expression of *Nr3c1* and *Crh* mRNA in the MBH prepubertal (P21) female rats as determined by qPCR (n=6/group). AU = arbitrary units. RNA expression data were normalized by dividing each individual value by the average of the control group at every time point. (b-c) Abundance of the TrxG-dependent activating marks H3K4me3 and H3K9ac and the PcG-dependent repressive mark H3K27me3 and H3K9me3 at the *Darpp32* and *Drd1* promoter in the prepubertal MBH of females perinatally exposed to a mixture of EDCs (F1 generation), as measured by ChIP (n=6/group). Bars represent mean ± s.e.m.

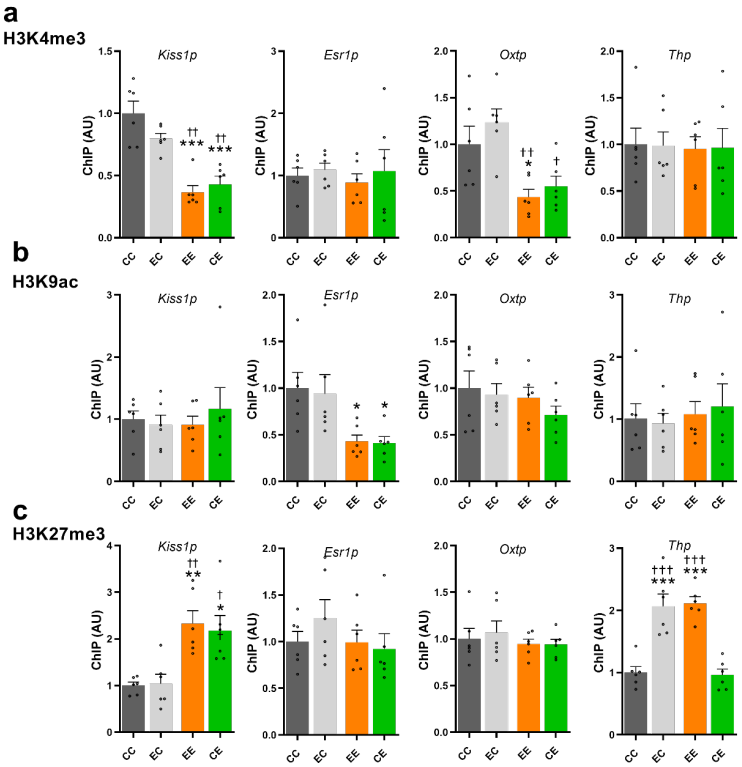

Figure E.10.Abundance of the TrxG-dependent activating marks H3K4me3 (a) and H3K9ac (b) and the PcG-dependent repressive mark H3K27me3 (c) at the *Kiss1*, *Esr1*, *Oxt* and *Th* promoter in the prepubertal (P21) MBH of cross-fostered germ-cell EDC exposed pups or control (F2 generation) raised by either *in utero* EDC exposed dams or control, as measured by ChIP (n=6/group). CC= control pup raised by control dam; EC: control pup raised by *in utero* EDC exposed dam; EE= germ-cell EDC exposed pup raised raised by *in utero* EDC exposed dam; CE= germ-cell EDC exposed pup raised by control dam. Bars represent mean ± s.e.m. (*P < 0.05, **P < 0.01 vs. CC, †P < 0.05 vs. CE, one-way ANOVA).

Gene		Primer	Accession number	Amplicon size	Use
*Kiss*1	F	*TGGTGAACCCTGAACCCACAGGC*	NM_181692.1	136	qPCR
	R	*CGGCGGGCATGGCGATGTT*			
*Esr*1	F	*CGCTCTGCCTTGATCACACA*	NM_012689.1	188	qPCR
	R	*GCCGAGGTACAGATTGGCTT*			
Oxt	F	*GCTGCCAGGAGGAGAACTAC*	NM_012996.3	175	qPCR
	R	*ATCATCACAAAGCGGGCTCA*			
Pomc	F	*CTTTCCGCGACAGAGCCT*	NM_139326.2	113	qPCR
	R	*CCAGCTCCACACGTCTATGG*			
Th	F	*CCTTCCAGTACAAGCACGGT*	NM_012740.3	109	qPCR
	R	*TGGGTAGCATAGAGGCCCTT*			
Cart	F	*GCGCTGTGTTGCAGATTGAA*	NM_017110.1	105	qPCR
	R	*CGTCACACATGGGGACTTGG*			
*Nr3c*1	F	*GGTGATTGAACCCGAGGTGT*	NM_012576.2	147	qPCR
	R	*TTTCTGAAGCCTGGTATCGCC*			
Crh	F	*CAAGGGAGGAGAAGAGAGCG*	NM_031019.1	160	qPCR
	R	*AAGAAATTCAACGGCTGCGG*			
*Grin*2*d*	F	*AGCTCTGCGACCTGCTGT*	NM_022797.1	190	qPCR
	R	*CCAAGCTGCAGGAAGGTGGA*			
*Dnm*1	F	*TCTACAAGGATTACCGGCAGC*	NM_080689.4	121	qPCR
	R	*GCTTTCTCCTTGTCCCCAACA*			
*Drd*1	F	*CCACTCTCCTGGGCAATACC*	XM_006253600.3	180	qPCR
	R	*AAAAGGACCCAAAGGGCCAA*			
*Darpp*32	F	*CCCAAGGACCGCAAGAAGAT*	NM_138521,1	172	qPCR
	R	*CTCCTGAGGTTCTCTGGTGC*			
*Grid*2	F	*GTCCCATCGAAAGAGGATGACA*	NM_024379.1	97	qPCR
	R	*ACTGTTTATGGGGGCTGTCG*			
Avp	F	*AGCGATGAGAGCTGCGTG*	NM_016992.2	129	qPCR
	R	*CTGTACCAGCCTAAGCAGCA*			
*Kiss*1	F	*TCGGGCAGCCAGATAGAGGAAGC*	NM_181692.1	91	ChIP
	R	*TTGAGGGCCGAGGGAGAAGAG*			
*Esr*1	F	*GTCCCTCAGCAGCCAGCCAGTCT*	NM_012689.1	127	ChIP
	R	*CTCTCGGGAAGCAGCCAGTAGG*			
Oxt	F	*TGTAGCTTAGGCCTCCCCTT*	NM_012996.3	159	ChIP
	R	*CATGACTGGTCACAGCAGGT*			
Pomc	F	*GCTAAGCCTCTGTCCAGTCC*	NM_139326.2	103	ChIP
	R	*GTTAGCACAGACCCGCTGAA*			
Th	F	*CCGACTGGGGCAGTGAATAG*	NM_012740.3	198	ChIP
	R	*TAACCAAACCAGGGCACACA*			
Cart	F	*TTCCATTTCATGGGCCCTCC*	NM_017110.1	139	ChIP
	R	*GGCTGGAGCACAGAGAACAA*			
*Nr3c*1	F	*AAGGGTTAGAAGGAATTTGGGGA*	NM_012576.2	180	ChIP
	R	*TGACGTGCCAGAGCCAATTA*			
Crh	F	*ACGCAATCGAGCTGTCAAGA*	NM_031019.1	96	ChIP
	R	*CAGAGCCCGGAGTGAGATTT*			
*Grin*2*d*	F	*TCTGGTTCTGTTCCTGGGTTTTTG*	NM_022797.1	121	ChIP
	R	*TGGGGTCAGGGAAGATACAGAGGT*			
Th − *BS*	F	*TCGTCGGCAGCGTCAGATGTGTATA*	NM_012740.3	302	BS-seq
		AGAGACAGGTTTTTTTTAGGTATAGTAGG			
	R	*GTCTCGTGGGCTCGGAGATGTGTATAA*			
		GAGACAGtatttatttataggtacaaaag			

Table E.1. Primer Sequence. F:forward, R:reverse

Target	Host	Source	Catalog #	Use
Th	Mouse	ImmunoStar	22941	IHC
H3K27me3	Rabbit	Active Motif	39155	ChIP
H3K9ac	Rabbit	Active Motif	39917	ChIP
H3K4me3	Rabbit	Active Motif	39159	ChIP
H3K9me3	Mouse	Active Motif	61013	ChIP
β-Galatocidase	Rabbit	Cortex Biochem	CR7001RP2	ChIP
β-Galatocidase	Mouse	ICN Biomedical	55976	ChIP

Table E.2. List of primary antibodies.

Measure	F	N	Average		SD		*p-value*	Effect Size	Figure
			CTL	EDC	CTL	EDC			
Vaginal Opening	F1	51/56	33,92	33,98	1,71	2,94		0,02	2a
	F2	50/52	34,73	37,93	1,33	1,58	0,000	2,19	2b
	F3	15/24	34,29	38,20	1,92	1,32	0,000		2c
	F4	47/64	31,36	34,50	1,36	1,40	0,000	2,27	2d
GnRH IP	F1	4	44,38	43,75	1,25	1,44			2a
	F3		42,03	44,06	0,94	1,08	0,030	2,01	2c
Regular cycle	F1	20	88,75	89,38	27,77	26,06		0,02	3a left
Proestrus			23,93	23,31	4,06	5,18		-0,13	
Estrus			26,58	28,78	6,16	12,48		0,22	
Diestrus			49,49	47,91	5,70	8,47		-0,22	
Regular cycle	F2	15	89,17	60,83	24,49	42,75	0,034	-0,81	3b left
Proestrus			23,75	22,50	3,30	4,13		-0,33	
Estrus			26,04	40,42	4,96	17,34	0,001	1,13	
Diestrus			50,21	37,08	5,60	15,12	0,002	-1,15	
Regular cycle	F3	15/14	90,83	49,11	22,89	45,85	0,004	-1,15	3c left
Proestrus			24,08	17,73	5,24	8,87	0,048	-0,87	
Estrus			26,29	37,79	1,70	12,59	0,000	1,28	
Diestrus			49,65	44,49	4,57	6,81	0,048	-0,89	
Folliculogenesis									3a middle
Primordial	F1	10/9	17,83	12,40	11,52	8,13		-0,55	
Primary			6,28	6,68	4,41	5,95		0,08	
Secondary			3,55	2,71	1,84	1,57		-0,49	
Antral			1,23	1,00	0,98	0,89		-0,25	
Atretic			8,42	16,25	3,87	11,70		0,90	
Corpora lutea			0,02	0,05	0,03	0,05		0,61	
Cysts			1,11	0,66	0,80	0,36		-0,73	
Folliculogenesis									3b middle
Primordial	F2	10/9	21,70	9,09	16,52	7,62	0,038	-0,98	
Primary			9,25	9,08	6,73	5,61		-0,03	
Secondary			8,54	7,16	6,50	4,14		-0,25	
Antral			2,55	0,74	1,16	0,88	0,045	-1,76	
Atretic			18,08	33,62	6,79	25,12	0,014	0,84	
Corpora lutea			0,01	0,12	0,03	0,16		0,97	
Cysts			0,63	1,13	1,06	1,36	0,470	0,41	
Folliculogenesis									3c middle
Primordial	F3	10/9	25,31	9,96	12,37	5,59	0,033	-1,60	
Primary			7,00	8,25	5,37	8,31		0,18	
Secondary			2,49	3,52	1,98	2,21		0,49	
Antral			1,20	0,29	0,85	0,37	0,048	-1,38	
Atretic			10,64	13,70	10,06	8,08	0,002	0,34	
Corpora lutea			0,04	0,07	0,08	0,12		0,27	
Cysts			1,13	1,02	0,60	1,31		-0,10	
Ovarian Weight	F1	14	47,69	40,33	7,34	9,94	0,035	-0,84	3a right
	F2	16	41,44	35,54	8,85	6,61	0,041	-0,76	3b right
	F3	10/9	65,24	44,67	10,90	7,96	0,000	-2,16	3c right

Table E.3. Report of descriptive and statistical data. Effect size was calculated using Cohen's *d*. F= generation. N=sample size. SD: standard deviation. IP: interpulse interval.

Measure	F	N	Average		SD		*p-value*	Effect	Figure
			CTL	EDC	CTL	EDC		Size	
Kiss1 P6	F3	6	1	0,39	0,13	0,23		-3,23	4a
Kiss1 P21			1	0,51	0,34	0,29	0,034	-1,56	
Kiss1 P70			1	0,38	0,07	0,34	0,026	-2,52	
Esr1 P6			1	0,85	0,15	0,24		-0,76	
Esr1 P21			1	0,32	0,50	0,32	0,019	-1,61	
Esr1 P70			1	0,21	0,07	0,16	0,013	-6,49	
Oxt P6			1	0,22	0,15	0,16		-4,95	
Oxt P21			1	0,23	1,26	0,19	0,018	-0,86	
Oxt P70			1	0,36	0,09	0,33	0,043	-2,64	
Cart P6			1	0,77	0,10	0,78		-0,41	
Cart P21			1	3,24	0,32	1,66	0,013	1,88	
Cart P70			1	0,87	0,16	0,43		-0,41	
Pomc P6			1	0,22	0,15	0,15		-5,24	
Pomc P21			1	2,60	0,15	1,38	0,023	1,63	
Pomc P70			1	1,00	0,17	0,75		0,01	
*Kiss1*p H3K27me3			1	10,15	0,34	6,78	0,008	1,91	4b
*Kiss1*p H3K4me3			1	0,22	0,46	0,09	0,002	-2,38	
*Esr1*p H3K9ac			1	0,32	0,69	0,20	0,044	-1,33	
*Oxt*p H3K4me3			1	0,32	0,69	0,20	0,044	-1,33	
*Pomc*p H3K4me3			1	0,38	0,51	0,21	0,021	-1,58	
Licking	F0	15	6,52	5,14	2,64	3,07		-0,49	5a
	F1	10/11	9,18	5,84	3,27	3,59	0,038	-0,97	5b
	F2	11	11,93	6,38	3,72	3,49	0,008	-1,54	5c
	F3	11	13,79	9,71	2,40	2,79	0,004	-1,57	5d
Resting alone	F0	15	0,57	0,24	1,09	0,35		-0,41	5a
	F1	10/11	0,68	1,93	0,93	1,07	0,011	1,24	5b
	F2	11	0,44	1,62	0,43	1,19	0,021	1,30	5c
	F3	11	2,85	3,18	3,20	3,14		0,10	5d
Th P6	F1	6	1	0,65	0,19	0,27		-1,51	6a
Th P21			1	0,18	0,64	0,10	0,031	-1,79	
Th P60			1	0,11	0,22	0,13	0,013	-4,93	
Dnm1 P6			1	0,30	0,24	0,14		-3,58	
Dnm1 P21			1	0,33	0,10	0,26	0,039	-3,45	
Dnm1 P60			1	1,15	0,29	0,66		0,30	
Drd1 P6			1	1,22	0,22	0,33		0,79	
Drd1 P21			1	3,41	0,24	1,70	0,016	1,99	
Drd1 P60			1	0,95	0,14	0,76		-0,09	
Darpp32 P6			1	0,75	0,17	0,29		-1,03	
Darpp32 P21			1	0,26	0,73	0,19		-1,39	
Darpp32 P60			1	0,19	0,30	0,09	0,002	-3,64	
*Th*p H3K27me3			1	3,12	0,52	2,10	0,019	1,39	6c
*Th*p H3K9me3			1	1,32	1,12	1,27		0,26	
*Th*p H3K4me3			1	1,19	0,27	0,77		0,33	
*Th*p H3K9ac			1	1,21	0,64	0,67		0,32	
Th-ir SN			92,7	87,64	21,05	21,15		-0,24	6e
Th-ir VTA			125,3	116,20	26,47	24,22		-0,36	
Th-ir mPoA			3,98	2,53	0,61	0,36	0,001	-2,90	
Th P6	F3		1	0,40	0,89	0,37		-0,88	6f
Th P21			1	0,36	0,16	0,17	0,040	-3,79	
Th P60			1	0,46	0,91	0,44		-0,77	
Drd1 P6			1	0,36	0,58	0,23	0,486	-1,47	
Drd1 P21			1	0,47	0,11	0,15	0,042	-4,11	
Drd1 P60			1	0,15	0,17	0,25	0,001	-3,93	
*Th*p H3K27me3			1	2,16	0,47	1,62		0,97	6h
*Th*p H3K9me3			1	2,17	0,38	1,13	0,037	1,38	
*Th*p H3K4me3			1	0,90	0,36	0,43		-0,26	
*Th*p H3K9ac			1	1,04	0,33	0,51		0,08	

Table E.4. Report of descriptive and statistical data. Effect size was calculated using Cohen's *d*. F= generation. N=sample size. SD: standard deviation. qPCR and ChIP data were normalized to the control group.

Measure	F	N	Group	Average	SD	Comparison	*p-value*	Effect Size	Figure
Vaginal opening	F2-C	16/19	CC	34,67	1,56	CC vs. CE	0,003	-1,29	7a
			CE	37,05	2,09	CC vs. EE	0,003	-1,46	
			EE	37,13	1,81	CE vs. EC	0,001	1,30	
			EC	34,72	1,45	EE vs. EC	0,001	1,47	
Regular cycle		10	CC	85,00	18,34				7b
			CE	75,00	27,50				
			EE	75,00	23,90				
			EC	88,33	17,66				
Proestrus			CC	21,25	3,65				
			CE	19,58	6,53				
			EE	22,08	4,83				
			EC	22,92	2,95				
Estrus			CC	23,33	3,51	CC vs. EE	0,008	-1,13	
			CE	25,83	6,15				
			EE	32,50	10,90				
			EC	26,25	4,41				
Diestrus			CC	55,42	6,23	CC vs. EE	0,002	1,30	
			CE	54,58	8,21	CE vs. EE	0,008	1,07	
			EE	45,42	8,88				
			EC	50,83	4,30				
Kiss1		6	CC	1,00	0,19	CC vs. CE	0,001	2,94	7c
			CE	0,46	0,18	CC vs. EE	0,012	2,03	
			EE	0,61	0,20	CE vs. EC	0,000	-2,96	
			EC	1,05	0,22	EE vs. EC	0,004	-2,13	
Esr1			CC	1,00	0,41	CC vs. CE	0,015	2,12	7d
			CE	0,36	0,12	CC vs. EE	0,038	1,84	
			EE	0,44	0,13	CE vs. EC	0,006	-2,03	
			EC	1,07	0,48	EE vs. EC	0,017	-1,79	
Oxt			CC	1,00	0,22	CC vs. CE	0,003	2,68	7e
			CE	0,42	0,22	CC vs. EE	0,005	2,87	
			EE	0,44	0,17	CE vs. EC	0,001	-2,17	
			EC	1,06	0,36	EE vs. EC	0,002	-2,22	
Th			CC	1,00	0,10	CC vs. EE	0,000	5,02	7f
			CE	1,14	0,26	CC vs. EC	0,001	3,42	
			EE	0,37	0,15	CE vs. EE	0,000	3,68	
			EC	0,52	0,17	CE vs. EC	0,000	2,83	
Kiss1	F3-C		CC	1,00	0,35	CC vs. CE	0,027	1,42	7c
			CE	0,56	0,26	CC vs. EE	0,043	1,47	
			EE	0,59	0,18	CE vs. EC	0,007	-2,53	
			EC	1,09	0,14	EE vs. EC	0,011	-3,08	
Esr1			CC	1,00	0,20	CC vs. CE	0,001	3,09	7d
			CE	0,29	0,26	CC vs. EE	0,002	3,15	
			EE	0,32	0,23	CE vs. EC	0,002	-2,15	
			EC	0,97	0,36	EE vs. EC	0,003	-2,12	
Oxt			CC	1,00	0,20	CE vs. EC	0,003	1,49	7e
			CE	0,57	0,11	EE vs. EC	0,003	2,63	
			EE	0,56	0,16				
			EC	1,27	0,52				
Th			CC	1,00	0,25	CC vs. EE	0,000	3,22	7f
			CE	1,00	0,22	CC vs. EC	0,000	2,89	
			EE	0,37	0,12	CE vs. EE	0,000	3,50	
			EC	0,32	0,22	CE vs. EC	0,000	3,05	
*Kiss1*p H3K27me3			CC	1,00	0,18	CC vs. EE	0,004	-2,69	7c
			CE	2,18	0,79	CC vs. CE	0,011	-2,05	
			EE	2,33	0,67	EE vs. EC	0,005	-2,17	
			EC	1,04	0,50	CE vs. EC	0,015	1,72	
Esr1 p H3K9ac			CC	1,00	0,41	CC vs. EE	0,046	1,81	7d
			CE	0,41	0,17	CC vs. CE	0,039	1,86	
			EE	0,43	0,17				
			EC	0,94	0,51				
Oxt p H3K4me3			CC	1,00	0,48	CC vs. EE	0,044	1,54	7e
			CE	0,55	0,27	EE vs. EC	0,003	2,78	
			EE	0,44	0,20	CE vs. EC	0,012	-2,19	
			EC	1,24	0,35				
Th p H3K27me3			CC	1,00	0,24	CC vs. EE	0,000	-4,46	7f
			CE	0,96	0,23	CC vs. CE	0,000	0,17	
			EE	2,11	0,26	EE vs. EC	0,000	-0,12	
			EC	2,07	0,48	CE vs. EC	0,000	-2,91	

Table E.5. Report of descriptive and statistical data. Effect size was calculated using Cohen's *d*. F= generation. N=sample size. SD: standard deviation. qPCR and ChIP data were normalized to the control group.

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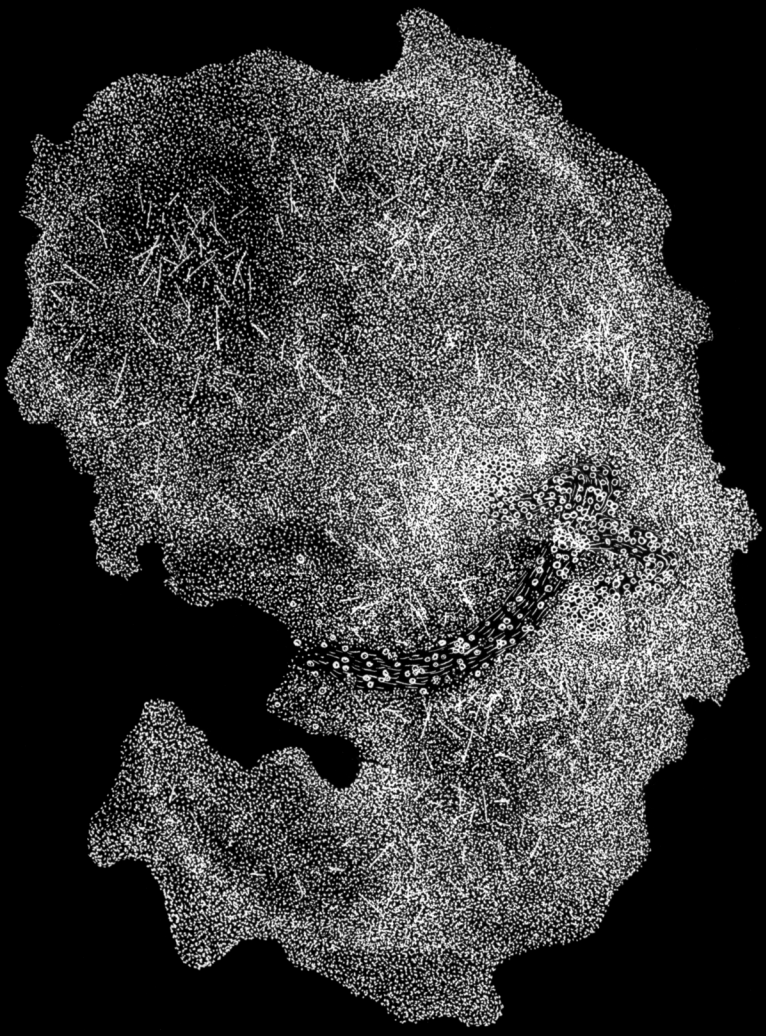

« The only principle that does not inhibit progress is: *anything goes.* »

Paul Feyerabend