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Review article

Mini-review: Epigenetic mechanisms that promote transgenerational actions of endocrine disrupting chemicals: Applications to behavioral neuroendocrinology

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ABSTRACT

It is our hope this mini-review will stimulate discussion and new research. Here we briefly examine the literature on transgenerational actions of endocrine disrupting chemicals (EDCs) on brain and behavior and their underlying epigenetic mechanisms including: DNA methylation, histone modifications, and non-coding RNAs. We stress that epigenetic modifications need to be examined in a synergistic manner, as they act together *in situ* on chromatin to change transcription. Next we highlight recent work from one of our laboratories (VGC). The data provide new evidence that the sperm genome is poised for transcription. In developing sperm, gene enhancers and promoters are accessible for transcription and these activating motifs are also found in preimplantation embryos. Thus, DNA modifications associated with transcription factors during fertilization, in primordial germ cells (PGCs), and/or during germ cell maturation may be passed to offspring. We discuss the implications of this model to EDC exposures and speculate on whether natural variation in hormone levels during fertilization and PGC migration may impart transgenerational effects on brain and behavior. Lastly we discuss how this mechanism could apply to neural sexual differentiation.

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1. Introduction

The organization/activation hypothesis is a core principle in the field of Behavioral Neuroendocrinology (Barraclough and Gorski, 1961; Kudwa et al., 2006; McCarthy et al., 2012; Phoenix et al., 1959). Its basic tenet is that gonadal hormones *organize* neural development acting on cell birth, migration, and/or death (Forger, 2016; McCarthy et al., 2009). In adulthood, gonadal hormones *activate* synaptic connections, signaling, and neuronal activity (Arnold and Gorski, 1984; McEwen et al., 1991). Since the first study demonstrating that administration of testosterone to neonatal female guinea pigs masculinizes sexually dimorphic reproductive behaviors (Phoenix et al., 1959) great strides have been made to understand both where and how gonadal hormones produce neural sex differences (McCarthy, 2019a, 2019b; McCarthy et al., 2012; Wright and McCarthy, 2009). The "how" question has become more refined over the years, as research has moved into the field of epigenetics (Forger, 2016).

In addition to hormones, a variety of environmental factors can affect sexually dimorphic endpoints. For example some natural plant products as well as man-made compounds have endocrine-like properties (Kudwa et al., 2007; Lephart et al., 2001; Patisaul et al., 2006, 2007). Endocrine disrupting chemicals (EDCs) are defined by the Endocrine Society as "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone signaling" (Zoeller et al., 2012). We are ubiquitously exposed to these compounds in construction materials, cosmetics, shampoo, pesticides, plastic food-storage containers, food cans, and a wide variety of other daily use products (Gore et al., 2019; Zoeller et al., 2012). The structure of EDCs make them more promiscuous than steroid hormones and they bind to more than one class of steroid receptors acting both as agonists and/or antagonists (Karthikeyan et al., 2019; McLachlan et al., 2012; Sharma et al., 2017). Some EDCs have been shown to modify the expression of genes involved in steroidogenesis and in this manner, they can change the endogenous concentrations of hormones (Bloom et al., 2016). There are now abundant data showing that fetal exposures to EDCs affect organization of sexually dimorphic brain regions and behavior in adults (Rebuli and Patisaul, 2016). Thus, in some cases these "hormone mimics" have usurped and/or derailed the roles of endogenous hormones.

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More than 30 years ago a team of cardiologists reported that underweight babies were more prone to develop cardiovascular diseases as compared to their normal-weight peers (Godfrey and Barker, 1995). These observations formed the basis of the theory of Developmental Origins of Health and Disease (DOHaD) (Heindel and Vandenberg, 2015; Gillman et al., 2007). The most intriguing DOHaD data show that early exposure to EDCs can have effects on subsequent generations, absent of direct contact with the chemicals (Jirtle and Skinner, 2007). The definition of a transgenerational effect is that it is present in generations produced after ancestral exposure to the pertinent environmental factor. In the case of fetal exposure, the fetal gametes (which produce the F2 generation) are directly exposed; thus, changes in first (F1) and F2 generations are multi- but not transgenerational (Jirtle and Skinner, 2007). The third generation (F3) and thereafter has the potential to demonstrate transgenerational effects. Many transgenerational effects of EDCs on brain, behavior, and reproduction have been documented (Anway et al., 2005; Latchney et al., 2018; Rattan and Flaws, 2019; Rissman and Adli, 2014).

Here we will review the EDC transgenerational literature focusing on brain and behavior. We will concentrate on data from rodent studies, which are simplest to compare to Behavioral Neuroendocrinology studies in rats and mice. Also, transgenerational epigenetic mechanisms are not well conserved in the animal kingdoms or even classes of vertebrates (Heard and Martienssen, 2014). We review four EDCs, which have received much public and scientific attention. These are the “weak estrogenic” compounds Bisphenol A (BPA) and polychlorinated bisphenyl (PCB), the “anti-androgenic” compound vinclozolin, and the best studied phthalate di-(2-ethylhexyl) phthalate (DEPH) which can be “androgenic” or “estrogenic”. After reviewing the existing data, focused on DNA methylation, we introduce recent work that represents a synthetic model incorporating multiple epigenetic mechanisms. This work, from the Corces laboratory, presents a more structural perspective of the sperm epigenome including roles for transcription factors (TFs) in 3D organization (Gold et al., 2018; Jung and Corces, 2019; Rowley et al., 2017). We believe this model can be applied to and advance the field of Behavioral Neuroendocrinology as we strive to understand how hormones are integrated with epigenetic mechanisms that regulate sex differences in brain and behavior.

2. Transgenerational actions of EDCs on reproduction and behavior

2.1. Vinclozolin

The first group to investigate transgenerational actions of an EDC was Dr. Michael Skinner's laboratory (Washington State University). Vinclozolin is an anti-fungal pesticide with anti-androgenic actions (Anway et al., 2005; Bisenius et al., 2006). The basic paradigm used rat dams exposed to vinclozolin during fetal germ cell migration (gestational days 10–15). During this time in gamete development, DNA methylation is being re-established and thus the genome is vulnerable to modification. The initial report showed that transgenerational exposure to vinclozolin had negative effects on sperm viability including decreased motility and numbers (Anway et al., 2005).

Vinclozolin also affects behavior in F3 rats whose progenitors were treated in the same manner described above. This effect is sex-specific; only F3 females discriminate between control and vinclozolin-lineage males, preferring males that lack a history of exposure. Similarly epigenetically imprinted males do not exhibit a preference. In other behavioral tests, F3 males display decreased anxiety-like behavior and preference for social novelty (Crews et al., 2012; Krishnan et al., 2019). Females had increased anxiety-like behavior (Skinner et al., 2008; Krishnan et al., 2019). These studies demonstrate that an EDC can promote sex differences and transgenerational alterations in the epigenome that might influence sexual selection (Crews et al., 2007).

Large data sets from F3 brains have been examined for individual candidate genes and pathway analyses (Skinner et al., 2008, 2014; Crews et al., 2012; Gillette et al., 2014). Several genes related to anxiety-like behaviors are differentially expressed in F3 brain and some effects are sex specific. In a study combining EDC ancestry and stress, in F3 rats, unstressed females had elevated estrogen receptor α (*Esr1)* mRNA in the CA3 section of the hippocampus and the basolateral amygdala (BLA) but decreased mRNA levels in CA1 (Gillette et al., 2014). Males from the vinclozolin lineage had elevated *Esr1* in BLA and the bed nucleus of the stria terminalis (BNST) with a reduction in the CA1. Androgen receptor (*Ar*) was lower in F3 vinclozolin lineage female BNST and elevated in CA3. In males only, *Ar* mRNA was reduced in the CA1 and the medial preoptic area (Gillette et al., 2015). Notably there are several effects of developmental exposure to vinclozolin on neural sex differences (Wolf et al., 2004; Bisenius et al., 2006).

2.2. Bisphenol A

Bisphenol A is one of the most heavily manufactured industrial chemicals used worldwide (Vandenberg et al., 2012). Its use has been reduced recently but it is still used for lining of food cans and thermal paper. Urinary levels are detectable in over 92% of Americans, in many water supplies and in wildlife (Flint et al., 2012; Kolpin et al., 2002; Santhi et al., 2012; Vandenberg et al., 2007). BPA is an EDC with multiple actions and binds to estrogen, androgen, thyroid, and other nuclear receptors (Chevalier et al., 2012; Moriyama et al., 2002; Nishizawa et al., 2003, 2005; Vandenberg et al., 2012). It can also modify steroidogenic enzymes (Akingbemi et al., 2004; Arase et al., 2011), and promote both hypo- and hyper- DNA methylation (Anderson et al., 2012; Dolinoy et al., 2007; Doshi et al., 2011). BPA exposure can change the timing of neuronal migration (Nakamura et al., 2007), and neurogenesis (Jang et al., 2012; Komada et al., 2012) in the developing brain. In adults, it can alter spine densities (Leranth et al., 2008) and dendritic growth (Shikimi et al., 2004). Correlational data report relationships between BPA concentrations in urine from pregnant women and subsequent social and anxiety behaviors in infants and young children (Braun et al., 2009, 2011; Yolton et al., 2011). Transgenerational actions (F3 and F4) of BPA on fertility have been demonstrated in rodents exposed *in utero* and testicular abnormalities are reported into the third (F3) generation in rats (Manikkam et al., 2012; Salian et al., 2009a, 2009b).

Results from one of our laboratories (EFR) were the first to demonstrate that human relevant doses of EDCs have transgenerational actions on brain and behavior (Wolstenholme et al., 2012). We evaluated effects of BPA in F1 mice exposed prenatally, and in the F2, F3 and F4 descendants. Contrary to the results in the F1 generation, in the F2 and F4 generations, social behaviors were increased in BPA-lineage mice. In two other studies, BPA exposure increased social investigation in male and female juvenile mice in a test of social recognition in the F1 and F3 generations (Wolstenholme et al., 2013, 2019). Furthermore, F3 juveniles failed to recognize a novel stimulus, indicating a possible deficit in their social memory. Notably, there were no sex differences, and this effect was only observed in F3 mice with maternal lineage exposure to BPA (Wolstenholme et al., 2019).

Relevant to behavior, a set of post synaptic density genes are differentially expressed in brains of male F3 BPA- *versus* control lineage embryos and adults (Wolstenholme et al., 2019). The non-coding RNA Maternally Imprinted 3 (*Meg3*) is also differentially expressed in F3 brains (Drobna et al., 2018). Several studies in F1 rats and mice have shown that BPA reduces sex differences in brain (Rebuli and Patisaul, 2016). Intriguingly, immunocytochemistry revealed that at the protein level, estrogen receptor α (ERα) was higher in the hypothalamic anterior ventral periventricular region in F3 female brains from BPA-lineage mice yet lower in the BNST (Goldsby et al., 2017). This is the first report of a transgenerational change in ERα protein in contrast to mRNA.

2.3. Phthalates

Phthalates are a class of industrial plasticizers commonly found in flexible plastic products, specifically polyvinyl chloride, food packaging, medical equipment, pill coatings, and synthetic flooring (Latini, 2005). DEHP is one of the most prevalent phthalate plasticizers. DEHP is able to cross the placenta (Singh et al., 1975), is absorbed through the skin (Hopf et al., 2014), and metabolites are found in breast milk (Kim et al., 2015). Following *in utero* exposure to DEHP, sperm count and mobility were reduced in F1 through F4 mice (Doyle et al., 2013). A mixture of phthalates given to neonates increased uterine weight, decreased anogenital distance, and caused fertility complications in the F3 generation (Zhou et al., 2017).

Previous studies in our laboratory (EFR) showed that transgenerational DEHP exposure modified nonsocial behaviors in F3 juvenile C57BL/6 J male mice (Quinnies et al., 2015). Pubertal F3 males from a DEHP-lineage showed increased digging and decreased self-grooming. Using a lower dose of DEHP, both male and female F3 juvenile mice exhibited modified social behavior (Quinnies et al., 2017) with no change in anxiety-like behavior. A recent study (Hatcher et al., 2019) found that transgenerational DEHP exposure had a sex-specific impact on anxiety-like behavior in F3 adult mice, decreasing anxiety in high-dose lineage females but not in males at any doses tested. They also observed changes in the expression of both nuclear estrogen receptor genes (*Esr1 and Esr2*), that were either up-or down-regulated in the amygdala in a dose- and sex-specific manner. Interestingly, in F3 females exposed ancestrally to DEHP, ERα (*Esr1*) mRNA was depressed; this is similar to the result in F3 BPA-lineage females but in a different part of the limbic system (Goldsby et al., 2017). This gene was also differentially expressed in vinclozolin-lineage brain, along with *Ar* (Gillette et al., 2015). The reports of transgenerational effects of EDCs on *Esr1* and *Ar* expression in brain could be important for epigenetic actions of these compounds.

2.4. Polychlorinated biphenyls

The polychlorinated biphenyls compounds (PCBs) are used in industrial chemicals and are widely present in the environment (Casati et al., 2015). Due to their toxicity and extremely long persistence, PCB production was forbidden by USA in 1979 and by the Stockholm Convention on Persistent Organic Pollutants in 2001 (Colciago et al., 2006) but they are still detectable in body tissues, umbilical cord blood and serum and urine (Gore et al., 2019).

Mennigen et al. (2018) described transgenerational effects of low doses of exposure to a PCB mixture. One of the most interesting findings was a significant increase in post weaning body weight in F3 mice, consistent with an “obesogen hypothesis” of EDCs. In addition, specific to the F3-maternal lineage females, serum progesterone concentrations in exposed rats were increased compared to controls (Mennigen et al., 2018). More recently, ultrasonic vocalizations in F3 rat pups were recorded and at the oldest age (post natal day 6) offspring from the PCB-lineage had reduced vocalizations (Krishnan et al., 2019). A PCB mixture given to pregnant (gestational day 10.5) mice in a genetic background susceptible to PCB actions (Hufgard et al., 2019) did not reveal differences in open field, learning in the water maze, or fear conditioning in F3 *versus* control adults.

3. Epigenetic transgenerational mechanisms

The assumed source of transgenerational alterations in brain and behavior is changes in gene expression achieved through epigenetic modifications. Another source could be genetic mutation, albeit most mutations lead to embryonic lethality. Vinclozolin acts on a variety of epigenetic endpoints but one study noted a copy number variation (CNV) in a candidate gene promoter in F3 rat sperm (Guerrero-Bosagna et al., 2010). A follow up study used kidney and sperm from *lacI* mutation-reporter rats (McCarrey et al., 2016). In the F1, exposed to vinclozolin *in utero,* no increase in mutation frequencies was observed, but by the F3 generation mutation frequencies in the vinclozolin lineage were elevated in both tissues. However, the *lacI* mutation-reporter rat is an indirect readout, which lacks sensitivity (whole genome sequencing would be the best method). While the assumption in the field is that epigenetic modifications and *not* changes in DNA sequence are responsible for transgenerational effects, we need to be clear that it is possible these compounds might act *via* mutations also.

3.1. DNA methylation

The best-studied epigenetic mechanism for EDC exposure and transgenerational changes is DNA methylation. The endpoint typically examined is differentially methylated regions (DMRs) in gene promoters. This mechanism has been emphasized for several reasons. First, the original work by the Skinner laboratory focused on DNA methylation (Anway et al., 2005). At that time DNA methylation, unlike histone modifications, were considered permanent. We now know that this is not true and that DNA demethylase enzymes can reverse DNA methylation (Wu and Zhang, 2017). BPA actions were noted using the agouti viable yellow mouse, an *in vivo* assay for DNA methylation status (Dolinoy et al., 2007). However, the effects are dose-dependent (Anderson et al., 2012) and have never been demonstrated past the F2 generation.

Vinclozolin changes DMRs in a variety of tissues, including sperm (Beck et al., 2017; Skinner et al., 2013, 2019; Gillette et al., 2018). On average about 250 DMRs were noted in F3 immature sperm at two embryonic ages with little overlap between the ages. Locations of the DMRs did not overlap with the differentially expressed genes. In adult sperm collected from F1 and F3 vinclozolin and control-lineages (Beck et al., 2017) DMR numbers in F1 sperm were lower than in F3 with little to no overlap. The DMRs identified were mainly present in regions of low CpG density. An independent group using a 100-fold lower dose of vinclozolin reported on F1 and F3 DNA methylation in mature sperm and brain (Gillette et al., 2018). One third of the DMR found in F1 and F3 sperm overlapped with each other. A comparison of DMRs in this study and the work from the Skinner laboratory would be very useful.

The report mentioned above (Gillette et al., 2018) included a concurrent study using the PCB Aroclor 1221 (A1221). Rat sperm, from A1221 lineages had twice the numbers of DMRs in F1 than in F3. Gene overlap between vinclozolin and A1221 was low (13–16 genes) but interestingly most of these were non-coding RNAs. Changes in DNA methylation in response to fetal (F1) BPA exposure have been observed for rats and mice in sperm and brain (Cheong et al., 2018; Doshi et al., 2012; Kundakovic et al., 2013; Susiarjo et al., 2013; Yaoi et al., 2008; Yin et al., 2016). A mixture of BPA and two phthalates given during mid-gestation to rats produced a variety of peripheral phenotypes in F3 animals (Manikkam et al., 2013). Because BPA modifies expression of imprinted genes (Zhang et al., 2012; Doshi et al., 2013; Kumamoto and Oshio, 2013) in our laboratory we examined RNA-seq data from F3 brain and performed DNA methylation with bisulfite- and pyrosequencing on the non-coding (nc) RNA, *Meg3*. Only small differences were found in the promoter of this ncRNA which suggests other epigenetic modifications lead to its differential expression. In sum, correlations between DMRs and transgenerational actions of several EDCs are known, but the genes themselves have not been rigorously investigated and causal data are lacking. Rigorous computational comparisons of DMRs, EDCs, and doses could be insightful. From this candidate genes could be collected and tested in material from PGC, mature sperm and brain in F1 and F3 individuals. Given the availability of CRISPR technology and lines of knockout mice, candidates could and should be tested. This would go far to pinpoint the epigenetic targets, but to really understand mechanisms we have to look beyond DNA methylation.

3.2. Other epigenetic mechanisms

DNA methylation is only one of many known epigenetic modifications. Since many genes do not contain CpG islands this mechanism cannot modify all genes. A few studies have examined transgenerational modifications in histones and/or non-coding (nc) RNAs as a result of developmental exposures to EDCs. The same paradigm discussed above was expanded to examine total histone 3 (H3) retention sites (DHR) (Chambers et al., 2011; Ben Maamar et al., 2018b) and RNA-seq for long (lnc) and ncRNAs (Schuster et al., 2016). Differences in both are present in F3 sperm from vinclozolin- *versus* control-lineage rats. In a separate study both these modifications and DNA methylation were probed in F1, F2 and F3 sperm (Ben Maamar et al., 2018a). The major conclusions were that the numbers of DMRs and DHRs increased between the F1 and F3 generations, while lncRNAs decreased and ncRNAs remained the same. Overlap between the marks was infrequent. In mice exposed ancestrally to BPA (as well as two replacement bisphenols) analysis of F3 sperm revealed differential expression of genes for histone-regulating enzymes (Shi et al., 2019). No differences in expression of H3K9me2 or H3K9me3 were found. To summarize 15 years of work in this field we have identified many DMRs but we are no closer to understanding mechanisms for transgenerational inheritance produced by EDCs. One large obstacle is that all the modifications discussed undergo extensive reprogramming during development thus it is not clear how these modifications are maintained over many generations.

4. Sperm contain transcription factors

The dogma that sperm are transcriptionally silent and therefore lack proteins involved in transcription has shaped the direction of this and other research fields. Recent work has challenged this notion and is reviewed here (Gold et al., 2018; Jung et al., 2017, 2019). Sperm DNA is highly methylated, and as such is a rich source of DMRs. The sperm genome is tightly packed, to a greater degree than in any other cell type. Despite this high degree of methylation, it appears that demethylated regions of DNA exist near promoters (Gold et al., 2018). One study showed that 30% of promoters containing H3K27me3 also had H3K4me2; these bivalent marks are indicative of a poised transcription state (Brykczynska et al., 2010; Sin et al., 2015) and reside largely in genes involved in embryonic development (Arpanahi et al., 2009; Weber et al., 2007). More sensitive methods reveal that approximately 60% of gene promoters are transcriptionally primed with nucleosomes containing activating histone modifications (Jung et al., 2017). A subset of the poised sperm promoters are in a transcriptionally active state, although not transcribed, and are retained in the post-fertilization inner cell mass. This makes it plausible that these modifications present in sperm can be inherited by the next generation. In addition to active histone modifications associated with many gene promoters, sperm chromatin contains H3K4me1 and H3K27ac in association with transcription-primed enhancer regions. The arrangement of these modifications is similar to that found in embryonic stem cells (ESCs).

An aspect of the sperm genome that has been under appreciated is its 3-dimensional (3D) structure. The transcription factor CCCTC-binding factor (CTCF), partners with the protein cohesin to mediate 3D organization of chromosomes. Sperm contain roughly 23,000 CTCF/cohesin sites (Jung et al., 2017), which contribute to the 3D architecture. This organization is also similar to ESCs. Sperm chromatin is organized into loops that contain either active or repressive epigenetic modifications (Jung et al., 2017). This aspect of the epigenome has been completely neglected in past studies of transgenerational inheritance.

A deeper examination of the transcriptional machinery in sperm shows many promoters are occupied by stable clusters of Mediator (a coactivator) and RNA polymerase II (Pol II) (Jung et al., 2019). These regions overlap with the active marks H3K4me3 and H3K27ac (Jung et al., 2019). Because the presence of Pol II in mature sperm does not correspond to the distribution of the transcription complex in round spermatids, it is possible that its presence in sperm represents a form of transcriptional priming to be activated after fertilization. Distal enhancers identified as transcriptionally primed are occupied by TFs including Pioneer factor Foxa1, ERα, and AR, a pattern conserved in human and monkey sperm. Both ERα and AR require Foxa1 for recruitment to chromatin (Gui et al., 2019; Hurtado et al., 2011). Interestingly, over half of the transcription start sites in sperm have all three proteins present. Similar but not identical and less detailed results are noted in oocytes. Taken together these data suggest that TFs and other proteins present in the gametes may remain bound to chromatin after fertilization and influence gene expression in the developing embryo. Very recent studies demonstrate that CpG sites bound by TFs during the re-methylation of germ cells are protected from methylation across development (Kremsky and Corces, 2019). Since many EDCs (including those reviewed here) bind to ERα and/or AR, if these EDCs are present in the embryo during the reprogramming of the germ line when the DNA is demethylated, it is possible that they could influence the recruitment patterns of TFs, including CTCF, to DNA. These bound TFs could then protect their new binding sites from remethylation. In fact, Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq) experiments carried out with sperm from the F1 through F5 progeny of mice exposed to BPA during germline reprogramming show the presence of new binding sites for several TFs, including CTCF, Foxa1, ERα and AR, at putative regulatory elements (Jung and Corces, unpublished results). If they remain bound after fertilization, these TFs could affect cell differentiation during development and elicit the phenotypes observed after EDC exposure. In addition, estradiol can enrich CTCF-binding to enhancer regions (Fiorito et al., 2016). If an EDC has that same effect, this is another potential avenue of EDC action. If these atypical patterns change the 3D landscape and/or the transcription of important developmental genes they can impact the F1 offspring. The maintenance of these new TF sites through several generations suggests the intriguing possibility that these alterations may be responsible for the phenotypic effects observed by initial exposure to EDCs but expressed generations later in their absence.

5. Transgenerational actions of hormones

Considering these data and the model described above we speculate that steroid hormones during normal development may likewise act on sperm DNA and could, in the same manner, produce transgenerational effects. It is well known that pharmaceuticals, like the potent estrogen agonist diethylstilbestrol, DES, can affect multiple generations. DES was prescribed to prevent miscarriages (which it failed to do) for a period of about 20 years. After a disproportional number of DES-daughters developed a rare reproductive cancer, clear cell adenocarcinoma, it was banned (Verloop et al., 2017). This created an opportunity to collect human transgenerational data from DES-children (F1) and grandchildren (F2), which revealed higher rates of reproductive cancers and structural malformations of the reproductive tract than in controls (Reed and Fenton, 2013; Tournaire et al., 2018). Behavioral effects (data from offspring of over 47,000 DES-treated women) are seen as well, including increased incidence of attention deficit hyperactivity disorder in DES-grandchildren (Kioumourtzoglou et al., 2018). Since exposure to DES was *in utero* the germ cells that became the F2 grandchildren received direct exposure. Truly transgenerational data will be collected in the next generation.

Given the DES data and other studies in rodents (Horan et al., 2017; Lv et al., 2018) it is plausible that steroid hormones may be agents for transgenerational actions, even under non-pharmacological conditions. Some examples might include unpredictably high levels of glucocorticoids produced by a variety of stressful conditions (Rodgers et al., 2015). Nutrition might also change hormone levels (*i.e.* phytoestrogens in Asian diets) which may have extended generational actions on sexual

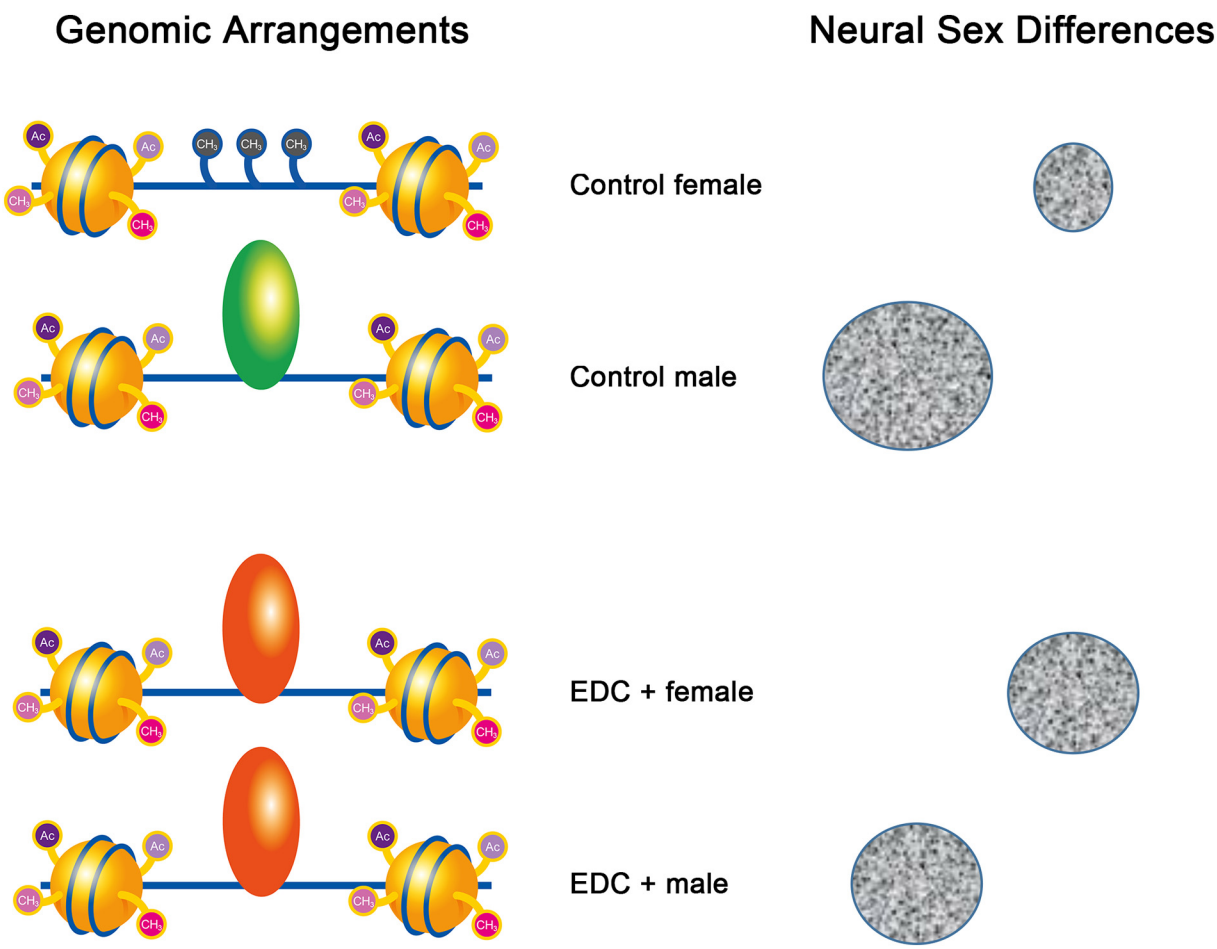

Fig. 1. Hypothetical adaptation of sperm-transcription factor (TF) arrangements in a sexually dimorphic brain region. The top two lines could represent control situations in normal rodent brain. The female genome shows DNA methylation and a small brain nucleus. The control male has retained a TF and has a larger sexually dimorphic nucleus. The bottom two lines demonstrate that after endocrine disruptor chemical (EDC) exposures both males and females have retained a TF that is different from the one in normal males. This produces equivalent sized nuclei. Ovals in green and orange are TFs, The circles with pixilation represent sexually dimorphic cell nuclei. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

behavior and/or reproductive outcomes (Guerrero-Bosagna and Skinner, 2014). Variations in testosterone levels within the normal range, for example in women with polycystic ovarian disease, could skew AR or ERα occupancy in developing PGC (Barrett et al., 2018). Intrauterine position (which influences hormone exposure) in rodents affects a number of phenotypes in the F1 pups (vom Saal and Bronson, 1980a, 1980b). We suggest that some of these hormonal modifications alone or in combinations might contribute to phenotypes in F2 or F3 offspring. Studies to test this hypothesis could reveal new mechanistic insights.

6. Epigenetics and neural sexual differentiation

Returning to a theme of this mini-review we suspect the model developed in sperm cells might translate to neurons and sexual differentiation. The basic paradigm is that fetal hormones act on brain just after gonadal differentiation, a period when the embryonic germ cells are undergoing re-methylation. In the past ten years a handful of studies have examined epigenetic mechanisms that might underlie this effect. One of our laboratories examined two histone modifications, H3K9/14 ac and H3K9me3 in brains of male and female mouse embryos and newborns (Tsai et al., 2009). In the cortex and hippocampus both these marks were sexually dimorphic (males > females) and the acetylation mark was masculinized in females treated with testosterone during gestation. In neonatal rat medial preoptic area, chromatin immunoprecipitation (ChIP) was performed with total histone 3 or 4 antibodies and the promoters of the ERα and aromatase genes probed (Matsuda et al., 2011). In embryos (E21) males had more H4 associated Esr1 and more H3 complexed with one of the aromatase enzyme promoters than did females. After birth (3 days of age) the reverse pattern was found. DNA methylation was quantified in two sexually dimorphic neural regions in mouse pups (4 days of age) and adults (Ghahramani et al., 2014). The largest sex differences were present on the X-chromosome, and notably, more sex differences were present in adult than in pup brains. A drug that blocks DNA methylation given to mouse pups at birth modified two sexually differentiated nuclei (Mosley et al., 2017). In the case of calbindin, which marks the sexually dimorphic nucleus in the medial preotic area the treatment increased numbers of cells demonstrating calbindin protein, but did not eliminate the sex difference. The other marker, estrogen receptor α was enhanced in male brain, and this eliminated the (M < F) sex difference.

Given these data and the important roles for *Ar* and *Esr1* in neural sexual differentiation we predict that use of ATAC-seq would be very enlightening (Fig. 1). Because *Foxa1, Esr1* and *Ar* are among the TFs retained on promoters in sperm we hypothesize these could be bound to steroids during normal sexual differentiation in brain. Using ATAC-seq, sex differences before and after brain differentiation may be revealed. Transcription factors at enhancers and promoters can be interpreted to decipher the nature of transcribed or silenced genes. At present, work in our laboratories is aimed at testing this hypothesis.

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