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Developmental exposure to bisphenol A alters expression and DNA methylation of *Fkbp5*, an important regulator of the stress response

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ABSTRACT

Bisphenol A (BPA), an abundant endocrine disruptor, affects stress-responsiveness and related behaviors in children. In rats, perinatal BPA exposure modifies stress response in pubertal offspring via unknown mechanisms. Here we examined possible epigenetic modifications in the glucocorticoid receptor gene and its regulator *Fkbp5* in hypothalamus and hippocampus of exposed offspring. We found increased DNA methylation of *Fkbp5* and reduced protein levels in the hippocampus of exposed male rats. Similar effects were obtained in a male hippocampal cell line when exposed to BPA during differentiation. The estrogen receptor (ER) antagonist ICI 182,780 or ERβ knock-down affected *Fkbp5* expression and methylation similarly to BPA. Further, BPA's effect on *Fkbp5* was abolished upon knock-down of ERβ, suggesting a role for this receptor in mediating BPA's effects on *Fkbp5*. These data demonstrate that developmental BPA exposure modifies *Fkbp5* methylation and expression in male rats, which may be related to its impact on stress responsiveness.

1. Introduction

Bisphenol A (BPA) is a widely used component of plastics and resins with endocrine disruptive features, exhibiting agonistic properties on both estrogen receptor (ER) isoforms, ERα and ERβ, and antagonistic properties on androgen receptor (Delfosse et al., 2014). Human exposure to this chemical is extensive since BPA is abundant in a vast number of consumer products, including toys, drinking bottles, food containers and dental sealants. Up to 95% of the human population has detectable BPA levels in their bodies and there is increasing concern for its higher bioaccumulation in developing organisms (Calafat et al., 2008). The reference dose of 50 μg BPA/kg of bw/day, previously determined as the safe daily human exposure (Vandenberg et al., 2012), has recently been reduced to 4 μg/kg of bw/day (http://www.efsa.europa.eu/en/topics/topic/bisphenol.htm) due to increasing evidence for adverse effects at lower exposures, especially impacting brain and prostate development in fetuses and children (http://www.niehs.nih.gov/health/topics/agents/sya-bpa/).

Early life exposure to BPA affects a variety of developmental functions (Kundakovic and Champagne, 2011), including neuronal differentiation and migration (Wolstenholme et al., 2012). In the rodent brain, BPA modifies sexual and social behavior, impairs cognition and increases anxiety and depression-like behavior (Wolstenholme et al., 2011). In our previous studies, we showed that perinatal exposure of rats to a low BPA dose alters their basal and stress-induced Hippocampal-Hypothalamic Pituitary Adrenal (HHPA) axis activity and related behaviors at mid-puberty in a sexually dimorphic manner (Poimenova et al., 2010; Panagiotidou et al., 2014): BPA-exposed females exhibited increased basal corticosterone and reduced hypothalamic glucocorticoid receptors (GR) levels, as well as anxiety-like behavior but less efficient late stress responses. BPA-exposed males, on the other hand, showed a heightened stress response compared to untreated counterparts. In humans, higher pre- and postnatal BPA levels have been associated with increased anxiety and depressive behavior in children, expressed differently between boys and girls (Braun et al., 2009; Braun et al., 2011; Harley et al., 2013; Evans et al., 2014). BPA crosses

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List of abbreviations

ANOVA	analysis of variance
BPA	bisphenol A
bw	body weight
ChIP:	chromatin immunoprecipitation
DCC	dextran coated charcoal
dex	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
E2	Estradiol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE-luc	estrogen responsive element-luciferase reporter
ERs	estrogen receptors
FBS	fetal bovine serum
Fkbp5	FK506 binding protein 5
FKBP51	FK506 binding protein 51
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HHPA	hippocampal-hypothalamic pituitary adrenal
ICI	ICI 182780
LSD	least square differences
Nr3c1	nuclear receptor subfamily 3, group C, member 1
PBS	phosphate-buffered saline
PMSF	phenylmethanesulfonyl fluoride
SDS	sodium dodecyl sulfate
shRNA	small hairpin RNA
ssDNA	single-stranded DNA

the placenta and although its metabolic clearance is high, its actions can be chronic and potentially engage epigenetic modifications. Indeed, BPA induces alterations in DNA methylation in various species, organs, and model systems upon different exposures (Kundakovic and Champagne, 2011).

The HHPA axis is an important regulator of the stress response and its dysfunction is correlated to several neuropsychiatric disorders including anxiety and depression. Glucocorticoids act as downstream effectors of the axis (Holsboer and Ising, 2010). By activating GR in the hippocampus and hypothalamus, glucocorticoids exert a negative feedback on the HHPA axis towards termination of stress response and resilience (Smith and Vale, 2006). GR function depends on a large complex of transcriptional co-regulators, chaperones and co-chaperones. One of them, FKBP51 (the protein product of the *Fkbp5* gene), reduces hormone binding affinity and nuclear translocation of GR (Riggs et al., 2003; Touma et al., 2011). *Fkbp5* is itself a GR target and glucocorticoids induce its expression as part of an intracellular ultra-short negative feedback loop for GR activity (Vermeer et al., 2003). Recent evidence indicates the sensitivity of *Fkbp5* to environmental factors and epigenetic changes, thus highlighting the importance of this co-regulator in stress related disorders (Schmidt et al., 2012). Chronic exposure to glucocorticoids persistently changes *Fkbp5* expression by altering DNA methylation of *Fkbp5* gene in the mouse hippocampus and hypothalamus (Lee et al., 2010; Yang et al., 2012; Wochnik et al., 2005). Interestingly, DNA methylation changes in the human *FKBP5* gene are also found in patients with post-traumatic stress disorder (Klengel et al., 2013a) and bipolar disorder (Fries et al., 2014).

Based on the above, we herein examined whether developmental exposure to BPA may lead to epigenetic alterations in genes encoding important mediators of the stress response, such as the glucocorticoid receptor and its regulator *Fkbp5*. Therefore we first investigated DNA methylation changes in the regulatory regions of the aforementioned genes in the hypothalamus and hippocampus of BPA-exposed rats. The detected changes in *Fkbp5* methylation in the hippocampus of male rats, which coincided with lower FKBP51 levels, led us to further examine the molecular basis of this BPA effect in a murine hippocampal cell line of male origin. Specifically, the involvement of estrogen receptors (ERs) in mediating BPA's effects in hippocampal neurons was analyzed by inhibiting ER using either ICI 182780 (ICI) or shRNA-mediated knock-down. Our results suggest an involvement of ERβ in BPA's epigenetic effects on *Fkbp5*.

2. Materials and methods

2.1. Chemicals

Bisphenol A and dexamethasone were purchased from Sigma–Aldrich (St. Louis, Missouri, USA), ICI 182780 from AstraZeneca (London, UK), cell culture reagents and Lipofectamine 2000 from Life Technologies (Carlsbad, CA, USA). Pre-designed shRNA against ERα and ERβ and control shRNA were obtained from Sigma–Aldrich. The luciferase reporter construct 3 × ERE-luc has been published (Legler et al., 1999). pRL-TK for normalisation of luciferase activity was purchased from Promega (Madison, WI, USA). Antibodies and primers used are listed in Supplemental material Tables S1 and S2.

2.2. Animals

Animal tissues used here were obtained in a previous study described elsewhere (Panagiotidou et al., 2014) and the protocol was approved by the Ethical Committee of the School of Health Sciences, National and Kapodistrian University of Athens, Greece. In brief, female Wistar rat breeders received BPA (40 μg BPA/kg bw/day) or the vehicle (water, 1% in ethanol) orally via impregnated cornflakes throughout pregnancy and lactation. The offspring (BPA-exposed or unexposed controls) were left to grow. At mid-puberty (postnatal day 46) the offspring were killed by decapitation either at basal conditions or two hours following a 15-min swimming stress.

2.3. Cell culture and treatments

The hippocampal cell line HT22, deriving from male mouse primary hippocampal cells (Liu et al., 2009), was cultured in Dulbecco's modified Eagle's medium (DMEM), supplied with 10% fetal bovine serum (FBS) and 0.5 mg/ml penicillin/streptomycin under standard conditions (37 °C, 5% CO_2).

For differentiation, HT22 cells were seeded into six-well plates (2×10^5 cells/well) in phenol red-free DMEM containing 5% dextran coated charcoal (DCC)-treated FBS and allowed to settle. Medium was changed to phenol red-free Neurobasal medium containing N_2 supplement and 100 μM dibutyrylcAMP, and different concentrations of BPA and/or 10 nM E2 or ICI 182,780 (ICI). After two days, medium was changed to phenol red-free Neurobasal medium without dibutyrylcAMP, BPA, and ICI, and allowed to grow for another 3 days. 1 μM dexamethasone was added 16 h before