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

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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.

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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 drinking bottles, food containers and dental sealants. Up to 95% of the human population has detectable BPA levels in their bodies and drinking bottles, food containers and dental sealants.

In children, developmental exposure to BPA alters expression and DNA methylation of Fkbp5, an important regulator of the stress response. 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.

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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% CO2).

For differentiation, HT22 cells were seeded into six-well plates (2 × 106 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 N2 supplement and 100 µM dibutyrylAMP, and different concentrations of BPA and/or 10 nM E2 or ICI 182780 (ICI). After two days, medium was changed to phenol red-free Neurobasal medium without dibutyrylAMP, BPA, and ICI, and allowed to grow for another 3 days. 1 µM dexamethasone was added 16 h before

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harvesting.

For ER knock-down studies, HT22 cells were seeded into six-well plates (2 × 10⁵ cells/well) in phenol red-free DMEM containing 5% DCC-treated FBS. The next day, mission shRNA was transfected using Lipofectamine 2000 according to the manufacturer’s instructions using 2.5 μg plasmid DNA and 4 μl reagent per well. After 24 h, medium was changed to phenol red-free Neurobasal medium containing N₂ supplement and 100 μM dibutyryl cAMP to induce differentiation.

2.4. Western blot analyses

Hippocampi were collected and immediately frozen in liquid nitrogen. The tissues (3–4 per animal group) were homogenized in NP-40 lysis buffer (137 mM NaCl, 20 mM Tris—HCl pH 8.0, 1% NP-40 (v/v), 10% glycerol (v/v), 48 mM NaF, 2 mM Na₃VO₄, and protease inhibitor mix). Similarly, whole cell extracts were prepared from treated or untreated cells homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris—HCl, pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 2 mM Na₃VO₄, and protease inhibitor mix). 50 μg or 30 μg total protein of the tissue extracts or whole cell extracts, respectively, were electrophoresed using 4%–12% polyacrylamide gels (Invi-

2.5. Gene expression analyses

RNA was isolated using Trizol (LifeTechnologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. 1 μg of total RNA was treated with DNasel and reverse transcribed using iScript (BioRad, California, USA). Real-time PCR was performed in a 15 μl reaction with 1 μl cDNA using SYBR® Select (LifeTechnologies, Carlsbad, CA, USA) and primers for Fkbp5, GR, ERS, and 36B4 (Supplemental material, Table S2) on an ABI 7500 Fast Real Time PCR System (Thermo Scientific, Waltham, Massachusetts, USA). Relative gene expression was assessed using the ΔΔCt method. 36B4 expression was chosen as most robust normalizer, compared to GAPDH, β-actin, and 18S RNA.

2.6. DNA methylation analyses

Genomic DNA was extracted from cells using GenElute™ (Sigma—Aldrich, St. Louis, Missouri, USA) and from rat brain tissues using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany) following manufacturer’s instructions. Bisulfite conversion was performed using EZ Methylation Gold Kit (Zymo Research, Irvine, California, USA) and Nr3c1 promoter, Fkbp5 intron 5 and intron 1was amplified by nested PCR (for primer sequences (Supplemental material, Table S1)). Pyrosequencing was performed with 25 μl of the resulting PCR product in a PyroMark Q96 ID using PyroMark Q96 Gold reagents (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

2.7. Luciferase assays

HT22 cells were seeded into 24-well plates (5 × 10⁴ cells/well) in phenol red-free DMEM containing 5% DCC-treated FBS. The next day, pRL-TK and 3 × ERE-luc were transfected using Lipofectamine 2000 according to the manufacturer’s instructions using a total of 0.5 μg plasmid DNA and 1 μl reagent per well. 9 h after transfection, fresh medium was added containing 10 nM E2 and/or 1 μM BPA. The next day, luciferase reporter assays were performed using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer’s protocol: cells were lysed in passive lysis buffer and firefly and renilla luciferase activity measured in 96-well plates using a Tecan infinite F200 luminometer (Tecan). Firefly luciferase activity was normalized to renilla luciferase activity.

2.8. Chromatin immunoprecipitation (ChiP)

ChiP assays were performed as described (Cortazar et al., 2011) 2–5 μg of the respective antibody was used (Supplemental material, Table S1). DNA was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and the isolated DNA fragments were analysed by qPCR using Rotor-Gene SYBR Green PCR Kit on a Rotor-Gene RG-3000 (Qiagen, Hilden, Germany).

2.9. Statistical analyses

To evaluate Western blot data, the mean ratio of FKBP51 to GAPDH or β-actin for each analysed sample was used. Two-way analysis of variance (ANOVA) was used to examine the effect of BPA treatment and stress on hippocampal FKBP51 levels. The LSD posthoc test was used for group comparisons. Unpaired student’s t-test was used for methylation, gene expression, and ChiP analyses unless stated otherwise. Significance was accepted for P < 0.05.

3. Results

3.1. Perinatal BPA exposure affects Fkbp5 methylation and FKBP51 levels in male rats

We have previously shown that perinatal exposure to a dose of BPA close to the range of human exposure leads to prolonged sex-specific alterations in stress response and HHPA-axis function in rats, affecting among others GR levels (Poimenova et al., 2010; Panagiotidou et al., 2014). Here, we set out to investigate if perinatal BPA exposure leads to DNA methylation changes in the GR gene (Nr3c1) and/or in its regulator Fkbp5. We used the hippocampi and hypothalamus from 46-day old rats whose mothers had been exposed to 40 μg BPA/kg bw/day during gestation and lactation, a dose belonging to the low-dose exposure range for BPA, repeatedly reported to exert adverse effects in experimental animals (Vandenberg et al., 2012), and investigated a region in the Nr3c1 promoter (Weaver et al., 2004) and the intron 5 of Fkbp5. No significant difference was found in the Nr3c1 promoter in either brain areas (Table 1), whereas in male hippocampi, we found a significant increase for methylation at Fkbp5 intron 5, an important regulatory region that is differentially methylated upon corticoid treatment (Lee et al., 2010) (Fig. 1a, Table 1). We also investigated methylation at Fkbp5 intron 1, another regulatory region of this gene (Lee et al., 2010), No significant differences were found in this region (Table 1).

Next, we examined if FKBP51 levels are affected in male hippocampi. At basal conditions FKBP51 protein levels were measured in 96-well plates using a Tecan infinite F200 luminometer (Tecan). Firefly luciferase activity was normalized to renilla luciferase activity.
Thus, perinatal BPA exposure leads to sexually dimorphic changes in hippocampal Fkbp5 methylation approximately one month after exposure has finished. At basal conditions, methylation changes coincide with reduced FKBP51 levels in the hippocampus of male offspring. However, following stress, FKBP51 levels are increased to levels of untreated controls.

### 3.2. BPA treatment of differentiating HT22 cells induces changes in Fkbp5 expression and methylation

To address the mechanisms underlying the expression and methylation changes of Fkbp5 in BPA-exposed male rats, we employed a cell model. We chose HT22 cells, a murine hippocampal cell line derived from male mouse primary hippocampal cells that can be differentiated from proliferating mitotic cells to cholinergic neurons displaying neurite outgrowth (Liu et al., 2009). To mimic developmental exposure in animals, these cells were treated with BPA during the 2-days differentiation procedure and subsequently kept without BPA for another 3 days (Fig. 2a) before analysis. BPA treatment decreased Fkbp5 gene expression in a dose-dependent fashion with significant changes at doses as low as 10 nM (Fig. 2b). This was also reflected at the protein level showing a significant decrease upon BPA treatment (Fig. 2c and Supplemental material, Fig. S1b).

We then analysed Fkbp5 methylation upon treatment with 10 and 1000 nM BPA at the region in intron 5 that is homologous to the regions investigated in the rats (Fig. 2d). We focused on the CpGs 5 and 6, corresponding to CpGs 6 and 7 in the rat, which are part of a ~200 bp region conserved between mouse and rat containing a GRE. BPA treatment led to an increase in methylation at these two CpGs, which reached significance at CpG 5 for both doses (Fig. 2e).

Thus, BPA exposure of HT22 cells during differentiation leads to increased DNA methylation and decreased expression of Fkbp5 3 days after BPA treatment was stopped, similarly to the changes observed in vivo.

### 3.3. BPA treatment in HT22 cells changes Fkbp5 inducibility upon glucocorticoid treatment

As part of the negative feedback regulation of the HPA-axis, GR, activated by elevated glucocorticoid levels during stress, increases Fkbp5 expression by binding to GREs in regulatory regions of the gene, among others the one in intron 5 (Hubler and Scammell, 2004). We thus analyzed if Fkbp5 inducibility by glucocorticoids changes upon BPA treatment in the cell model. To this end, we differentiated HT22 cells in the absence and presence of 10 and 100 nM BPA at the region in intron 5 that is homologous to the rat model.

We used 1 μM dex, a concentration commonly used in cell experiments, e.g. (Klengel et al., 2013b), that induces Fkbp5 expression (Nehme et al., 2009). As expected, Fkbp5 expression increased upon dex-treatment in the absence of BPA (Fig. 3, left grey vs. black bar). Similar inducibility with 10 and 100 nM dex were observed (data not shown). In cells exposed to BPA during differentiation, basal Fkbp5 was decreased significantly whereas upon dex-stimulation, the increase of Fkbp5 expression was even higher than in the absence of BPA (Fig. 3, middle and right grey vs. black bars). Thus, BPA treatment during HT22 differentiation changes inducibility of Fkbp5 in response to glucocorticoids, similarly to the rat model.

### Figs.

**Fig. 1.** Perinatal exposure to BPA induces expression and DNA methylation changes of Fkbp51 in rat hippocampus. a: DNA methylation at Fkbp5 intron 5 in male rat hippocampus assessed by bisulphite-pyrosequencing. b: Quantification of western blot analyses of Fkbp51 protein levels in male rat hippocampus at basal conditions and 2 h after a 15 min-swimming stress. All bars represent the means of at least 3 animals, analysed in 3 independent experiments. *p < 0.05, **p < 0.01 BPA-treated vs. untreated, †p < 0.05 basal vs. stressed.

### Table 1

Percentage DNA methylation of Nr3c1 (glucocorticoid receptor) and Fkbp5 in hypothalamus and hippocampus assessed with bisulphite-pyrosequencing, BPA vs. control. Values that differ significantly (p < 0.05) between BPA and control treated are in bold.

| Gene      | Sex | CpG position | 1     | 2     | 3     | 4     | 5     | 6     | 7     |
|-----------|-----|--------------|-------|-------|-------|-------|-------|-------|-------|
| Hypothalamic | m   | 1.67/2.40    | 1.20/1.68 | 0.50/0.00 | 1.47/1.73 | 1.22/1.36 | 1.27/1.59 | 1.80/2.04 |
| Fkbp5 intron 5 | f   | 1.96/2.49    | 1.43/1.87 | 0.17/0.03 | 1.63/1.72 | 1.35/2.26 | 1.36/1.57 | 2.00/2.08 |
| Hippocampal | m   | 14.30/15.63  | 12.26/13.13 | 20.60/19.49 | 14.89/18.56 | 10.15/9.69 | 18.60/19.12 |
| Nr3c1      | f   | 14.75/14.68  | 11.05/11.96 | 19.11/19.25 | 19.21/19.43 | 10.45/10.40 | 20.07/18.68 |
| Hippocampal | m   | 0.30/0.42    | 1.04/1.26 | 1.61/0.80 | 1.41/1.62 | 1.59/1.32 | 1.62/1.51 | 1.49/1.90 |
| Nr3c1      | f   | 0.74/0.15    | 1.40/0.99 | 1.77/1.71 | 1.59/1.26 | 2.63/2.44 | 1.69/1.72 | 1.42/1.95 |
| Hippocampal | m   | 20.97/14.38  | 17.01/12.58 | 12.08/6.36 | 18.40/10.88 | 18.28/14.00 | 9.21/6.20 | 15.22/9.33 |
| Fkbp5 intron 5 | f   | 13.64/18.07  | 15.73/18.19 | 5.96/9.01 | 11.07/14.69 | 15.21/18.78 | 5.62/8.11 | 9.50/11.94 |
| Hippocampal | m   | 85.61/85.94  | 93.77/93.87 | 51.99/48.94 |
| Fkbp5 intron 1 | f   | 87.55/85.75  | 95.61/92.98 | 59.80/58.00 |
We tested if estradiol (E2) treatment during differentiation affects \textit{Fkbp5} expression, and if inhibition of ERs with ICI 182 780 (ICI) changes the effects of BPA. HT22 cells were differentiated as above in the absence or presence of 10 nM E2 or 1 \text{mM} ICI, which are commonly used doses of the hormone and antagonist (e.g. (Dauvois et al., 1992)). Gene expression was assessed 3 days after the differentiation was finished and the substances had been washed out (Fig. 4a). ICI treatment led to a decrease in \textit{Fkbp5} expression (Fig. 4a, left grey vs. black bar), similar to that observed upon BPA treatment (Fig. 4a, middle black bars). In accordance, FKBP51 protein levels were decreased by both BPA and ICI treatment and their combination (Fig. 4b, Supplemental Fig. S1c). E2, on the other hand, did not affect \textit{Fkbp5} expression by itself, but reverted the decrease induced by ICI (Fig. 4a, bars to the right).

We also tested if these treatments affect \textit{Fkbp5} expression through direct transcriptional activation. Acute treatment with BPA or E2 in the absence or presence of ICI for 6 h did not induce any differences in \textit{Fkbp5} expression (Fig. 4c). On the other hand, E2 readily activated an ERE-luciferase reporter construct transiently transfected into HT22 cells (Fig. 4d), demonstrating that ERE-driven transcription can be activated in these cells. BPA had no effect on luciferase activity by itself but reverted E2 induced transcription (Fig. 4d).

As ICI treatment had similar effects to BPA on \textit{Fkbp5} transcription, we also investigated its effects on DNA methylation at CpG 5 and 6. As shown in Fig. 4e, ICI increased \textit{Fkbp5} methylation to a similar extent as BPA. In the presence of both ICI and BPA during differentiation, \textit{Fkbp5} methylation increased even further, in particular for 1 \text{mM} BPA at CpG6 (Fig. 4e, middle and right grey vs. black bars). Thus, the ER inhibitor ICI affects \textit{Fkbp5} methylation and expression similarly to BPA, suggesting an involvement of the ERs in BPA’s effects, however, not via a classical ERE-driven transcriptional mechanism.
3.5. ERβ is involved in the effects of BPA on Fkbp5

To better understand the role of the ERs in regulating Fkbp5 transcription, we investigated if ERα and/or ERβ is recruited to the regulatory region of Fkbp5 at intron 5. To this end, we conducted chromatin immunoprecipitation assays in HT22 cells, analyzing ERα and ERβ binding to the differentially methylated region in intron 5 upon treatment with BPA and ICI. As shown in Fig. 5a, ERβ was significantly enriched at this region, both in the absence and presence of E2. On the other hand, no significant enrichment could be found for ERα with two different antibodies (data not shown). Upon ICI treatment, ERβ binding to intron 5 was lost (Fig. 5a), supporting a specific enrichment at this region. Following BPA treatment, ERβ recruitment decreased significantly compared to control (Fig. 5a).

We then tested a direct involvement of ERβ in mediating BPA’s effect on Fkbp5. Using shRNA, we knocked-down ERβ before induction of the differentiation process (Fig. 5b). Knock-down of ERβ lead to a significant decrease in Fkbp5 expression (Fig. 5c black bars, left panel) and to a significant increase in Fkbp5 methylation (Fig. 5c black bars, right panel). BPA treatment during differentiation decreased expression and increased methylation when cells were transfected with control shRNA. However, no further effect was observed when ERβ was knocked-down (Fig. 5c grey bars).

Together, these findings implicate that ERβ is involved in the effects of BPA on Fkbp5 transcription and methylation.

4. Discussion

The HHPA axis is essential for the organism to cope with stress. Dysfunction of this system has been associated with a number of psychiatric illnesses such as major depression and post-traumatic stress disorder. While major research efforts have been made to identify genetic components involved in psychiatric diseases, much less is known about environmental factors contributing to these disorders and the underlying mechanisms. In this study, we demonstrate both in an in vivo and in an in vitro model that BPA, an abundant chemical that disrupts estrogen signaling, can induce transcriptional changes in the regulation of Fkbp5, an important regulator of the HHPA axis.

We show that perinatal exposure to a low, human-relevant, BPA dose (Vandenberg et al., 2012) leads to epigenetic changes in the Fkbp5 gene in the hippocampus of male rats about one month after BPA exposure had finished. Increased methylation coincided with reduced FKBP51 levels at basal conditions that imply an increased alertness towards threatening insults. Notably, upon stress, FKBP51 levels of these animals increase, suggesting that BPA-treated male rats become equally efficient as controls to terminate an acute stress response. This competency complies well with previous results where BPA-treated males exhibited similar behavioral coping and corticosterone responses to stress as the untreated animals (Panagiotidou et al., 2014). The finding that a short-term stress can modify FKBP51 levels despite the BPA-induced methylation changes, suggests an enhanced expression plasticity of this regulator that was also observed in the HT22 cell line deriving from male mouse hippocampus.

Our findings in male rats are in good agreement with the results obtained in HT22 cells and demonstrate that this cell line is a valid model to study the mechanisms underlying the effects of BPA exposure on Fkbp5 regulation. In the cell line, BPA exposure during differentiation lead to decreased Fkbp5 expression and increased methylation at the corresponding CpGs in intron 5. Of note, these changes were not due to acute effects of BPA as they were observed 3 days after BPA washout. Similarly to the rat model, BPA’s effect on Fkbp5/FKBP51 levels was larger than on Fkbp5 methylation. This is at least partly due to the fact that the detection methods do not have the same sensitivity, which makes comparison of the results difficult. Further, other factors and/or genomic regions not investigated here but involved in Fkbp5 regulation might be affected by BPA.

In conclusion, dexamethasone treatment simulating the stressful situation in the rat model, increased Fkbp5 expression to a greater extend in BPA-treated than in the untreated cells. The reason for decreased basal expression of Fkbp5 but increased inducibility of the gene by glucocorticoids despite an increase in DNA methylation is not solved. One possibility is that methylation changes at the responsive element under study affect mineralocorticoid receptor binding, responsible for mediating the effects of basal glucocorticoid levels, but not GR binding that follows the increase of hormone levels. Although sharing the DNA recognition sequence, the two receptors are modulated in their activity by diverse co-activators and co-repressors that might be affected differently by the methylation changes. Further studies are necessary to understand the detailed implications of the DNA methylation changes induced by BPA.

The ER inhibitor ICI affected Fkbp5 expression and methylation during HT22 differentiation similarly to BPA. Furthermore, ERβ, but not ERα, bound to the differentially methylated region at intron 5, and this binding was disrupted by BPA and ICI. These results imply a function of ERβ in mediating BPA’s effects on Fkbp5, which was further supported by the fact that ERβ knock-down abolished the effects of BPA on Fkbp5 expression and DNA methylation. Notably, however, the effects found here cannot be ascribed to BPA’s estrogenic properties as i) they were observed three days after BPA had been washed out of the culture and ii) BPA did not display agonistic properties in the HT22 cells on an ERE-driven transcription, in contrast to E2 (Fig. 4c). Further, there is an agreement in the literature that BPA does not induce the same conformational changes as the natural ligands when binding to ERs (Wetherill et al., 2007; Delfosse et al., 2012), hence presumably attracting a different set of co-regulatory proteins than in the presence of natural ligand. Accordingly, we propose that BPA affects Fkbp5 transcriptional regulation by interfering with ERβ binding to the regulatory region of intron 5, where ERβ controls DNA methylation, a function of ERβ that we have described previously (Ruegg et al., 2011). A tentative model is depicted in Supplemental material, Fig. S3. We could not see any effect of BPA on ER or GR protein expression in our cell model (Supplemental material, Fig. S2b and c). Further, ERβ levels were not changed in the hippocampus of the treated rats (Supplemental material, Fig. S2d). However, in mice it was shown that BPA exposure leads to decreased ERβ levels in brain areas other than the hippocampus (Cao et al., 2014). Thus in other regions BPA...
might not affect DNA binding of ERβ but rather its protein levels. Ultimately, however, this will lead to the same result, a lack of ERβ binding to intron 5 of Fkbp5 and thus an increase in DNA methylation.

Interestingly, BPA seems to affect ERβ expression in the rodent brain in a sexual dimorphic manner (Cao et al., 2014) and data not shown). This might explain why we could not detect any methylation changes in female rats at the investigated regions of Nr3c1 and Fkbp5. Sexually dimorphic effects of BPA in brain function have been reported in several previous in vivo studies (Cao et al., 2014; Cao et al., 2013; Chen et al., 2014; Gioiosa et al., 2013; Jasarevic et al., 2013; Wolstenholme et al., 2011; Xu et al., 2015). Furthermore, the few epidemiological studies linking BPA exposure to neuropsychiatric outcomes in children also show differences
between girls and boys (Braun et al., 2011; Harley et al., 2013; Evans et al., 2014). This demonstrates the intricate interaction between BPA and the endogenous sex hormones and consequently the importance to investigate its effects on both sexes.

5. Conclusion

We demonstrate here that perinatal exposure of rats to a low BPA dose alters Fkbp5 expression, methylation pattern and inducibility by stress in the hippocampus of male offspring. The observed alterations in Fkbp5 were also detected in differentiating hippocampal neurons of male origin. In the cell model, the mechanism implicates ERβ in the regulation of the epigenetic impact, a finding that requires further studies in the in vivo setting. The BPA-induced changes in hippocampal Fkbp5 confer a link between environmental chemicals and stress-related disorders.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2015.09.028.

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