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Bisphenol A Exposure at Puberty Disrupts Expression of the Oestrogen Receptor-Alpha in the Hypothalamus of Male and Female Mice

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Research Article

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ABSTRACT

Bisphenol A is a xenoestrogen that interacts with both of the oestrogen receptors, oestrogen receptor alpha (ERα) and oestrogen receptor beta (ERβ). At environmentally low doses, bisphenol A has been reported to influence behaviour and affect development of the brain and reproductive tissues in rodents. In the present study mice were treated daily with a low (50 μg/kg) oral dose of bisphenol A starting on postnatal day (PND) 32 until sacrifice (PND50 or PND100), and at autopsy brain, testis, and uterus of all animals were harvested for analysis. Immunohistochemistry was used to evaluate the changes in the number of cells expressing ERα in the arcuate nucleus and ventromedial nucleus of the hypothalamus. Histological evaluations were performed on the testis and uterus, while analysis of estradiol and testosterone was carried out on mouse serum. At PND50, bisphenol A exposure increased ERα expressing neurons in the arcuate nucleus and ventromedial nucleus of the hypothalamus of both male and female mice ($p<0.05$), while at PND100, bisphenol A exposure only increased ERα expressing neurons in the ventromedial nucleus of the hypothalamus of female mice ($p<0.05$). No adverse effects were observed in the testis of exposed males, except for consistent thickening of the basement membrane. In the females, mild simple hyperplasia of the endometrial glands was observed with no differences between BPA50 and BPA100 groups. Male mice had decreased testosterone and elevated estradiol serum concentrations at PND50 while females were observed to have increased serum estradiol at PND100. Our results show that bisphenol A can modulate oestrogen receptors in the hypothalamus and indicate a comparable effect of exposure in male and female mice during adolescence but a differential effect during adulthood.

INTRODUCTION

Oestrogen is crucial in the development and sexual differentiation of central nervous system structures controlling neuroendocrine, behavioural and cognitive functions [1]. At puberty the organisation of neural circuits reaches full maturation,

whereby the increase in gonadal hormones is responsible for masculinisation and defeminisation of the male reproductive system and sexual behaviour [2, 3]. The effects of the sex steroid hormone oestrogen are mediated largely through oestrogen receptor-alpha (ERα) and oestrogen receptor-beta (ERβ) [4]. The ERα and ERβ steroid receptors are members of the ligand-activated nuclear superfamily that specifically bind to oestrogen and oestrogen-like chemicals and are able to regulate gene transcription. In the absence of oestrogen, ER exists in an inactivated and untransformed state, as a monomer bound to heat shock proteins [5]. Although the hypothalamus is a classical target area for oestrogen [6], it has been found that brain cells such as neurons, astrocytes, and oligodendrocytes also express many of the known ERs [7].

In rodents, ERα is highly expressed in regions that regulate reproductive function and behaviour, including the anteroventral periventricular nucleus, medial pre-optic nucleus, arcuate nucleus and ventromedial hypothalamus [8-10]. Although males and females are born with the same number of neurons in the brain region, it has been shown that estradiol-mediated selective cell death through the binding of ERα [11] during neonatal life rapidly induces morphological sexual differences [12]. Bisphenol A (BPA) is a monomer used in the manufacture of polycarbonate, epoxy and polystyrene resins which are used as materials in the production of food containers. In recent years, there has been an increase in the frequency of human exposure to BPA due to increasing use of polycarbonate plastics and epoxy resins [13]. Many studies have also indicated leakage of BPA traces from polycarbonate containers [14, 15] and epoxy linings in food containers [16, 17]. Bisphenol A is an endocrine disruptor that possesses oestrogen-like effects and can mimic the action of natural oestrogens in humans, which can cause reproductive and developmental toxicity [18]. Although BPA has reduced affinity to bind to ERs as compared to oestrogen, nevertheless it mediates its action mainly through this pathway [19].

In rodents, early life BPA exposure has been shown to impact both male reproductive behaviour [20] and female fertility [21]; effects consistent with hypothalamus perturbations. Therefore, the aim of this present study was to investigate whether exposure to BPA during puberty can change the expression of ERα in the short (PND50) and long (PND100) term. We performed a quantitative stereological study of the proportion of cells that express nuclear ERα in male and female mice and compared differences between the control and BPA exposed mice. This latter comparison enabled us to determine whether ERα expression in the brain was altered in the presence of BPA during puberty and throughout adulthood of the organism. We focused our analysis on the arcuate nucleus (ARC) and ventromedial nucleus (VMH), which are involved in reproductive behaviour for both sexes [22], and expression of oestrogen modulated ER [23].

MATERIALS AND METHODS

Animals and experimental design

Thirty six post-weaning male and female BALB/c mice were obtained from the Institute of Medical Research (IMR), Kuala Lumpur, Malaysia. After their acclimatization to the local animal house for five days, animals were randomly housed into groups of six, separated by sex and given food and water ad libitum. In the present experiment, mice were exposed in the beginning stages of puberty with 50 µg/kg body weight (BW) of BPA daily while mice in the control group received the vehicle (0.01% ethanol). The substances (BPA or ethanol) were delivered orally through a micropipette until the day prior to sacrifice. This route of exposure was chosen because it is more relevant to human exposure. This dose of BPA was chosen because it is well below the published "No Observed Adverse Effective Level" (NOAEL) dose of 5 mg/kg body-weight (bw)/day [24] and is the current established tolerable daily intake (TDI) for BPA [25, 26]. Additionally, numerous studies have reported the effects of BPA exposure at doses well below 5 mg/kg bw/day, and sometimes around only a few µg/kg bw/day [27]. Animals were sacrificed on either postnatal day (PND) 50 or PND100; additionally females were sacrificed during the estrus phase of the oestrus cycle. Accordingly, the exposed mice were identified as BPA50 (PND50) or BPA100 (PND100) and Control50 (PND50) or Control100 (PND100), respectively. All of the animal procedures were subjected to review and approved by the Research and Ethics Committee of the International Medical University (IMU). The procedures were in compliance with the National Institutes of Health (NIH) guidelines for humane treatment of laboratory animals.

Tissue collection and preparation

Animals were deeply anesthetized with diethyl ether and sacrificed by cervical dislocation. At autopsy, the brain from each animal was removed and post-fixed in 10% formaldehyde for 24 hours at room temperature. Uterus from females and testis from males were also harvested and fixed in a similar way. For tissue processing, coronal sections of the brain were cut from approximately bregma-1.0 through bregma-3.2. This region has been shown to contain the ARC and VMH. Adipose tissue was removed from the female reproductive tract and the testis was sliced longitudinally prior to processing. Fixed tissues were dehydrated in an ascending series of ethanol, cleared in xylene, and embedded in paraffin.

Immunohistochemistry

In each processed brain, two sections were sliced using a microtome in 5 µm frontal sections in the coronal plane, mounted on silane-coated slides and dried for 24 hours at room temperature. A standard immunohistochemical technique (avidin-biotin-peroxidase) was used to visualize the distribution of the immunostaining. Briefly, samples were subjected to pre-treatment for antigen retrieval, followed by blocking against endogenous peroxidase activity and nonspecific binding sites. Samples were then

incubated with primary antibody (anti-oestrogen receptor-alpha antibody sourced from Abcam) at a dilution of 1:100 at room temperature for 1 hour, and subsequently with a biotinylated secondary antibody for 30 minutes. The reaction was developed using 3,3'diaminobzidinetetrahydrochloride (DAB) as a chromogen substrate resulting in a brown reaction product. Samples were dehydrated and mounted with a permanent mounting medium.

Histologic analysis

Transverse sections of the uterus and testis were sliced at 5 µm, mounted on slides and dried at room temperature. Sections were stained with haematoxylin and eosin (H&E) and mounted with DPX, a non-aqueous mounting medium for microscopy containing mixed isomers of xylene and dibutyl phthalate.

Quantification of image analysis

Within each experimental group (n=6), brains were analysed and ERα immunoreactive cells with a clear brown stain were counted in each field by an investigator who was not blinded to the sex and treatment conditions. The number of positive cells in each section was scored and averaged per animal to obtain one number per animal. Manual cell counting was performed with a Nikon microscope equipped with a camera, by using ×20 objective lens. The scoring was assessed on the percentage of cells that expressed the brown colour using an established method describe in previous studies [28, 29], where the score was done based on the number of cells that took up the stain in one high power field. Selected brain sections to be analysed included the hypothalamic areas (ARC and VMH), with reference to the high resolution mouse brain atlas [30]. Histopathological evaluations were performed on the testis and uterus of all treatment groups.

Hormone assay

Whole blood collected at time of sacrifice was allowed to clot then centrifuged at 3000rpm for a period of 15 minutes at 4°C. The serum was pipetted out and stored in clean tubes at -80°C until use. Serum testosterone and estradiol concentrations were determined by competitive ELISA using commercially available kits produced by Cayman Chemical Company. Prior to the ELISA procedure for testosterone analysis, mouse serum was extracted with diethyl ether, following the instructions provided by the producer.

Data analysis

For the histological analysis, we compared incidence in each dose group with that in the control group using one-sided Fisher's exact test. One way ANOVA analyses were performed for the immunohistochemistry and hormone results to obtain the overall significance, and differences between treatment groups were determined using the Bonferonni post-hoc test. In all cases, $p<0.05$ was accepted as significant. All data were expressed as the mean ± S.E.M.

RESULTS

Immunohistochemistry

In the ARC of experimental animals, ERα expression was significantly increased by almost two-fold in BPA50 male mice ($p<0.05$) **(Figure 1)**, while there was a three-fold increase in ERα expression in the VMH of male animals in the BPA50 group ($p<0.001$). There were no differences observed in the ERα expression of control male mice for both regions at 50 and 100 days, while the differences in expression for BPA exposed mice were eliminated for both regions when the males reached 100 days old. BPA50 female mice had a marginal increase in ERα expression in the ARC and a significant increase ($p<0.001$) in ERα expression in the VMH. The same trend was observed for females in the BPA100 group. The number of cells expressing ERα in the younger control female mice was significantly lower as compared to the older females from the control group for both regions of the ARC ($p<0.001$) and VMH ($p<0.005$), respectively **(Figure 1).** Expression of ERα in the VMH and ARC in selected photomicrographs for male and female animals are presented in **(Figure 2).**

Histopathology

There were no macroscopic defects observed in the reproductive tract of the male animals studied. Most of the male mice in the treatment groups did show some evidence of morphological changes or differences in the testicular histology when compared with the control group **(Figure 3).** All mice in the control group showed the normal pattern of the spermatogenic cycle. In the treated groups, the seminiferous tubules showed the normal architecture with adequate cellularity with all forms of cells being present. There were also healthy quantities of Sertoli cells and Leydig cells present. However, most animals in the treatment groups showed mild thickening of the basement membrane in tubules, and there were correspondingly lower distribution of germ cells. This thickening was determined by morphological methods, comparing with normal tissue, which qualitatively allows a grading as mild, moderate and severe, based on established methods described in previous studies [31]. As with the other analysis, there was no difference observed between the two BPA treatment groups. Histological analysis revealed a thin muscular myometrial wall enclosing the endometrium in the uterus. The endometrium showed numerous glands arranged in a cellular compact stroma with no evidence of atypia in the glands of the control mice. However, after two months, glands showed elongation and increase in size, lined by crowded cells with prominent basal nuclei and moderate amounts of cytoplasm in animals from both BPA treated groups **(Figure 3).**

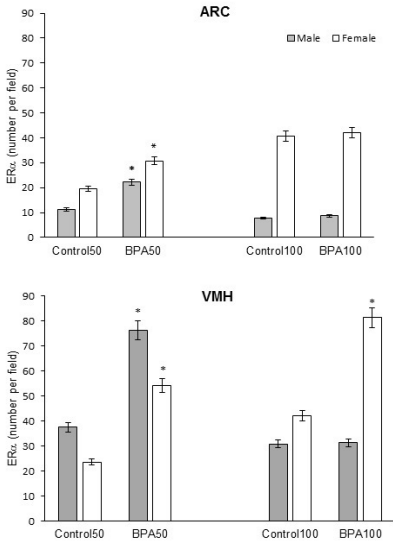

Figure 1. Effects of administration of vehicle and BPA to male and female mice during puberty until adulthood on ERα labelled neurons in the two hypothalamic areas: ARC, arcuate nucleus; VMH, ventromedial nucleus. Data are mean±SEM. * p<0.05 vs control group of the same sex.

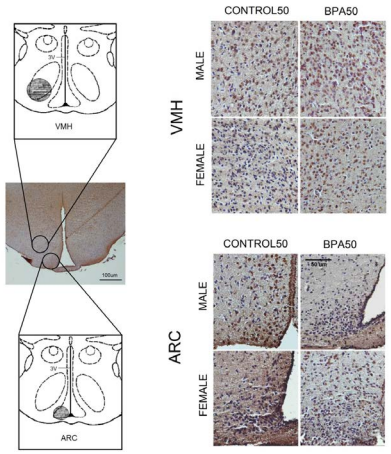

Figure 2. Photomicrographs showing examples of immunohistochemical staining of ERα in the arcuate nucleus (ARC, left) and ventromedial nucleus of the hypothalamus (VMH, right) of male and female mice (original magnification 100X).

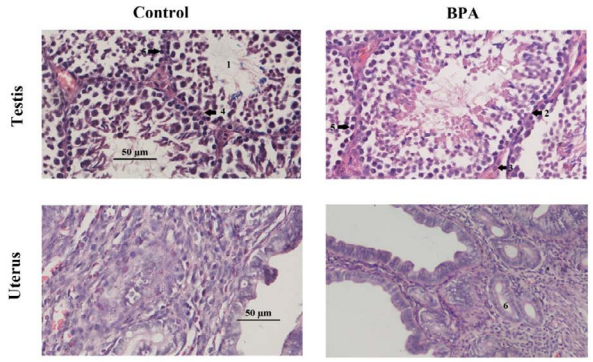

Figure 3. H&E staining of the testis (original magnification 200X) in male mice and uterus (original magnification 200X) in female mice. (1) lumen of seminiferous tubule, (2) basement membrane, (3) Leydig cell, (4) Sertoli cell, (5) spermatogonia, (6) mild hyperplasia of the endometrial glands, note the irregularly shaped glands.

With reference to uterine abnormalities, we observed simple hyperplasia of the endometrial glands with no differences between BPA50 and BPA100 groups, while no incidence of hyperplasia was observed in the control group **(Table 1).** There was no dose related variation between the two BPA groups and the differences between the control and BPA groups were statistically significant by Fisher's exact tests (p = 0.033).

Hormone assay

Testosterone serum levels for males were significantly lower for the BPA50 group when compared to the Control50. In males of the 100 day cohort, no significant difference was observed between the Control and the BPA exposed group. In females, there was no significant difference between all treatment groups. ANOVA revealed the significance of sex, due to the higher

levels in males than in females. Estradiol levels were significantly increased in the serum of BPA50 male mice. While there was no significant alterations of oestrogens observed with BPA100 male mice, an upward trend was apparent. In female mice, a significantly increased serum estradiol level in the adult mouse was observed in the BPA100 group. Testosterone and estradiol concentrations in males and females after treatment with BPA are presented in **(Figure 4).**

Table 1. Abnormalities found in mice treated with BPA.

Sex	Treatment	Mild basement thickening in testis	Mild hyperplasia of endometrium glands
Male	Control50	16.60%	-
	Control100	0%	-
	BPA50	50.0%*	-
	BPA100	66.6%*	-
Female	Control50	-	0%
	Control100	-	0%
	BPA50	-	60%*
	BPA100	-	40%*
* Significantly different at p<0.05 vs corresponding control			

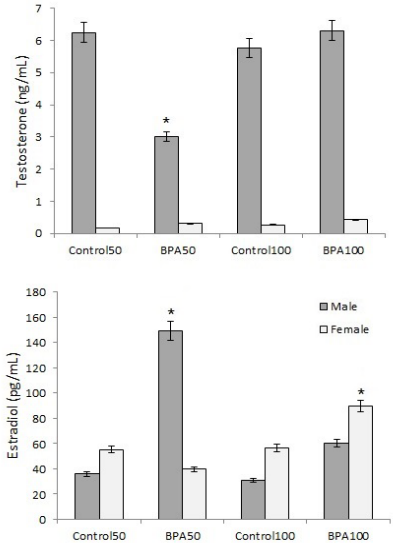

Figure 4. Effects of administration of vehicle and BPA to male and female mice during puberty and throughout adulthood on serum testosterone and estradiol levels. Data are mean±SEM; * p<0.05 *versus* control group of the same sex.

DISCUSSION

ERα expression

Relatively little research has been conducted in experimental animals to assess their effects on the CNS in sensitive periods such as puberty. We observed variations in ERα expression in the different regions of the ARC and VMH. The numbers of hypothalamic cells expressing ERα were modified in the BPA exposed groups and these were sex-dependent and dissimilar in the short and long-term. In the present study, Control50 males showed higher ERα levels than control females in the VMH, while no difference was observed in the ARC. This difference was absent in the VMH of Control100 males and females, while the levels in the ARC were one-fold higher in females and one-fold lower in males as a response to hormone priming. Interestingly, our results showed that, BPA exposure during the pubertal period had an overall effect in the ARC and VMH showing higher levels of ERα as compared to the controls independent of sex in the short term. Consequently, there was active alteration of ERα expression in the brain areas, which was dependent on the period of growth and the sex of the subjects [32].

BPA exposure during the early postnatal period has been shown to induce an upregulation in total ERα mRNA levels, mediated by means of an increased expression of hypothalamic ERα transcript variants and protein [33]. Additionally in early neonatal life, studies have shown elevated ERα levels in females [34] and an increase on the number of ERα-immunoreactive cells in the adult VMNvl [35] following BPA exposure. An increase in ERα and ERβ expression in juvenile rats has also been seen following neonatal BPA exposure [36].

These findings suggest that short term exposure to estrogenic substances during puberty can modulate oestrogen receptors in the hypothalamus. In animals sacrificed on PND100, females had higher ERα levels than males in all groups for both the ARC and VMH areas. BPA exposure did not modify the ERα levels in the ARC and VMH in males, but markedly increased it in the VMH of females. There is a fundamental difference between the male and female VMH in adulthood causing estradiol induced changes in the VMH more robust in females than males [37]. The present results suggest a similar effect of BPA exposure in male and female

mice during early puberty but a differential effect during adulthood. The aim of the present study was to assess whether exposure to BPA during puberty affects ERα in the short and/or long term. In the short term, there was essentially one main effect of BPA exposure which was an increase in ERα expression independent of sex in the ARC and VMH. In the long term, BPA had an effect only in the VMH of females. Thus, BPA given at puberty and throughout adulthood seems to permanently modify the level of ERα in the VMH of the female mouse brain.

Hormone alterations

In the present study, we aimed to assess whether ERα expression levels in the hypothalamus can be linked to effects on the reproductive system and hormonal balance disruption in serum. The effects of BPA were moderate, whereby we observed significant decreased levels of testosterone in BPA50 males, while estradiol levels were significantly increased. The dramatic decrease in testosterone concentration observed in male mice (BPA50) suggests that the testes may be a primary target organ of BPA exposure. In other studies, administration of BPA to male rats have resulted in decreased testosterone levels [38, 39] as well as a decline in androgens and activity of Leydig cells [39-41]. This corresponds to our observation in males having low serum testosterone level and elevated estradiol levels. Although, oestrogen is essential for normal male fertility, excessive production of these hormones could produce adverse outcomes, affecting the male reproductive system development and functions. We hypothesized that BPA stimulated gonadal steroidogenesis causing an increase in oestrogen, similarly to other studies which have implied that endocrine-disrupting chemicals could act to disrupt androgen synthesis and secretion by directly inhibiting enzymes in the testosterone biosynthesis pathway or by indirectly altering pituitary function [42, 43].

In BPA100 females, there was an increase in serum estradiol levels suggesting a peripheral effect for increased estradiol synthesis. Various studies have shown that, exposure to BPA increased estradiol and aromatase activity along with aromatase mRNA levels [43, 44]. Other studies report that BPA exposure alter the HPG axis by precocious hypothalamic–pituitary maturation leading to altered GnRH pulsatility in neonates and adults, and severe effects on GnRH signalling in the pituitary [45]. It is also unclear why BPA exposure did not affect estradiol and testosterone levels in the adult male mice in the BPA100 group in the current study. These findings reflect the complexity of the endocrine system, the numerous loops and the need to investigate different endpoints levels [43]. It has been postulated that ERα is critical for the organization of reproductive neuroendocrine pathways however the role which is played by ERα in the hypothalamus has not been fully elucidated [46]. Nevertheless, for both males and females it is challenging to declare that the differences observed in the ERα expression levels in the hypothalamus are responsible for the changes observed in hormone serum levels.

Histopathology

Pathological effects of the treatments were found in both BPA50 and BPA100 animals for testis and uterus. Under microscopic investigations, a dosage of 50 μg/kg BW did not have adverse effects on the morphology of the testis; nevertheless a reduction of germ cells was observed. A loss of germ cells has also been previously reported in the testis of rats treated with 500 μg of BPA and 17-β-estradiol [47]. Experimental evidences suggest that BPA adversely affects the male reproductive system in general and testis in particular [48], interrupting normal testicular function, inhibition of spermatogenesis and testicular testosterone secretion [27]. It has also been reported that BPA may stimulate some cellular responses similar to estradiol and influence multiple endocrine-related pathways.

In female animals the long term effects of BPA administration, which includes ovarian cystadenomas, squamous metaplasia, adenomyosis, leiomyomas, atypical hyperplasia and stromal polyps of the uterus and carcinogenesis, may be due to increased proliferation mediated indirectly by ERα in the stroma [49, 50]. In the current study, we observed mild hyperplasia in the endometrial glands of BPA exposed female. Furthermore, BPA has been shown to cause an increase in ERα expression in the lumina typifying a hyper-estrogenic response of the uterus [51]. Although we did not study ERα expression in the uterus, an increase in ERα expression in the VMH and increased serum estradiol levels were observed in adult female mice. Therefore, we suggest the incidence of simple hyperplasia maybe a consequence of misregulation in endocrine signalling pathways caused by the interaction of the latter two. Nevertheless, we did not find any case of malignancy since the animals in our experimental setting were exposed at critically low doses. The animals were also sacrificed at three months of age and probably had not enough time to fully develop tumours of the genital tract. Our current rodent studies have demonstrated that exposure to even low doses of BPA during the pubertal period of reproductive development and maturation can lead to subtle reproductive outcomes in male and female mice.

CONCLUSION

These results support the hypothesis that exposure to BPA during the pubertal period can impact the neuronal environment, leading to reproductive modifications. The processes of sexual differentiation and adult reproductive function are under genetic control and hormone-mediated feedback mechanisms. Most importantly, these processes are sensitive to regulation by endogenous and exogenous factors, including a list of environmental endocrine disruptors. Hence these findings should be a cause for concern regarding public health, confirming that exposure to low doses of weak environmental oestrogens during the stages of sexual maturation could influence adult reproductive performance. These findings emphasize the need for further studies aimed at uncovering the mechanisms by which exposure to environmentally disrupting chemicals impact the health

of exposed individuals during different development phases. Furthermore, some of the long-term effects observed in animals exposed to environmentally low doses of BPA, is deemed as acceptable by the U.S. Environmental Protection Agency [52, 53] and the European Commission Scientific Committee on Food [54, 55]. Further studies are necessary to elucidate the precise BPA actions that contribute to these anomalies during the pubertal period.

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REFERENCES

1. Arnold AP and Gorski RA. Gonadal steroid induction of structural sex differences in the central nervous system. Annual Reviews of Neuroscience. 1984;7:413-42.
2. Primus RJ and Kellogg CK. Gonadal hormones during puberty organize environment-related social interaction in the male rat. Hormones and Behaviour. 1990;24:311-323.
3. Sharpe RM, et al. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environmental Health Perspectives. 1995;103:1136-43.
4. McEwen BS and Alves SE. Estrogen actions in the central nervous system. Endocrine Reviews. 1990;20:279-307.
5. Jensen EV, et al. Estrogen binding substances of target tissues. Steroids. 1969;13:417-27.
6. Rai AL and Jeswar U. Immunolocalization of estrogen receptor alpha in adult female rat hippocampus. International J Morphology. 2010;28(2):483-7.
7. Azcoitia I, et al. Estradiol synthesis within the human brain. Neuroscience. 2011;191:139-47.
8. Shughrue PJ, et al. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. J Comparative Neurology. 1997;388:507-25.
9. Chakraborty TR, et al. Colocalization and hormone regulation of estrogen receptor alpha and NMDA receptor in the hypothalamus of female rats. Endocrinology. 2003;144:299-305.
10. Polston EK and Simerly RB. Ontogeny of projections from the anteroventral periventricular nucleus of the hypothalamus in the female rat. J Comparative Neurology. 2006;495:122-32.
11. Patchev AV, et al. Differential role of estrogen receptor isoforms in sex specific brain organization. FASEB J. 2004;18:1568-70.
12. Wright CL, et al. Cellular mechanisms of estradiol mediated sexual differentiation of the brain. Trends in Endocrinology and Metabolism. 2010;21:553-61.
13. Carwile L, et al. Polycarbonate bottle use and urinary bisphenol A concentrations. Environmental Health Perspectives. 2009;117:1368-72.
14. Nerin C, et al. Determination of bisphenol-type contaminants from food packaging materials in aqueous foods by solid-phase microextraction–high-performance liquid chromatography. J Chromatography A. 2002;963(1-2):375-80.
15. Brede C, et al. Increased migration levels of bisphenol A from polycarbonate baby bottles after dishwashing, boiling and brushing. Food Additives & Contaminants. 2003;20:684-9.
16. Yoshida T, et al. Determination of bisphenol A in canned vegetables and fruit by high performance liquid chromatography. Food Additives & Contamination. 2001;18:69-75.
17. Braunrath R, et al. Sample preparation including sol–gel immunoaffinity chromatography for determination of bisphenol A in canned beverages, fruits and vegetables. Journal of Chromatography A. 2005;1062:189-198.
18. NTP –CERHR Expert Panel report on the reproductive and developmental toxicity of bisphenol A. Developmental and Reproductive Toxicology. 2007.
19. Matthews JB, et al. In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta. Chemical Research in Toxicology. 2001;14(2):149-57.
20. Jones BA, et al. Pre- and postnatal bisphenolA treatment results in persistent deficits in the sexual behavior of male rats, but not female rats, in adulthood. Hormones and Behaviour. 2011;59:246-51.
21. Cabaton NJ, et al. Perinatal exposure to environmentally relevant levels of bisphenol A decreases fertility and fecundity in CD-1 mice. Environmental Health Perspectives. 2011;119:547-52.
22. Goldman BD. The circadian timing system and reproduction in mammals. Steroids. 1999;64:679-85.
23. Kudwa AE, et al. Roles of estrogen receptors alpha and beta in differentiation of mouse sexual behavior. Neuroscience. 2006;138:921-8.

24. NTP. Report of the endocrine disruptors low dose peer review. US department of health and human services. National Toxicology Program, Research Triangle Park, NC. 2001.
25. EFSA. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the Commission related to 2,2-BIS(4-HYDROXYPHENYL)PROPANE (Bisphenol A). The EFSA Journal. 2006;428:1-75.
26. USFDA. Draft assessment of bisphenol A for use in food contact applications. 2008.
27. Richter CA, et al. In vivo effects of bisphenol A in laboratory rodent studies. Reproductive Toxicology. 2007;24:199-224.
28. Chakravarthi S, et al. Apoptosis and expression of BCL-2 in cyclosporine induced renal damage and its reversal by beneficial effects of 4,5,7-Trihydroxyflavone. J Analytical Bio-Science. 2009;32(4):320-7.
29. Chakravarthi S, et al. 3A study of the significance of apoptosis and its association with abnormalities in expression of BCL-2 proto-oncogene in benign nodular hyperplasia of prostate. Research J Biological Sciences. 2009;4(7):836-1.
30. Sidman RL, et al. High resolution mouse brain atlas. 2013.
31. Haw KY, et al. Effects of Etlingeraelatior extracts on lead acetate-induced testicular damage: A morphological and biochemical study. Experimental and Therapeutic Medicine. 2012;3:99-104.
32. Ceccarelli I, et al. Estrogenic chemicals at puberty change ERα in the hypothalamus of male and female rats. Neurotoxicology and Teratology. 2001;29:108-15.
33. Monje L, et al. Neonatal exposure to bisphenol A modifies the abundance of estrogen receptor *a* transcripts with alternative 5 -untranslated regions in the female rat preoptic area. J Endocrinology. 2007;194;201-12.
34. Cao XL, et al. Bisphenol A in human placental and fetal liver tissues collected from Greater Montreal area (Quebec) during 1998–2008. Chemosphere. 2012;89:505-11.
35. Adewale HB, et al. The impact of neonatal bisphenol-A exposure on sexually dimorphic hypothalamic nuclei in the female rat. Neurotoxicology. 2011;32:38-49.
36. Ramos JG, et al. Bisphenol A induces both transient and permanent histofunctional alterations of the hypothalamic-pituitary-gonadal axis in prenatally exposed male rats. Endocrinology. 2003;144;3206-15.
37. Flanagan-Cato LM. Sex differences in the neural circuit that mediates female sexual receptivity. Frontiers in Neuroendocrinology. 2011;32:124-36.
38. Tohei A, et al. Bisphenol A inhibits testicular functions and increases luteinizing hormone secretion in adult male rats. Experimental Biology and Medicine. 2001;226:216-21.
39. Nakamura D, et al. Bisphenol A may cause testosterone reduction by adversely affecting both testis and pituitary systems similar to estradiol. Toxicology Letters. 2010;194:16-25.
40. Shan LX, et al. Immunohistochemical analysis of androgen effects on androgen receptor expression in developing Leydig and Sertoli cells. Endocrinology. 1997;138:1259-66.
41. Akingbemi BT, et al. Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology. 2004;145:592-603.
42. Shi Z, et al. Alterations in gene expression and testosterone synthesis in the testes of male rats exposed to perfluorododecanoic acid. Toxicological Science. 2007;98:206-15.
43. Quignot N, et al. Characterization of endocrine-disrupting chemicals based on hormonal balance disruption in male and female adult rats. Reproductive Toxicology. 2012;33:339-52.
44. Arase S, et al. Endocrine disrupter bisphenol A increases in situ estrogen production in the mouse urogenital sinus. Biology of Reproduction. 2011;84:734-42.
45. Fernandez M, et al. Neonatal exposure to bisphenol A alters reproductive parameters and gonadotropin releasing hormone signaling in female rats. Environmental Health Perspectives. 2009;117:757-62.
46. Rissman EF. Roles of oestrogen receptors alpha and beta in behavioural neuroendocrinology: beyond Yin/Yang. J Neuroendocrinology. 2008;20:873-9.
47. DCruz SC, et al. Bisphenol A impairs insulin signaling and glucose homeostasis and decreases steroidogenesis in rat testis: An in vivo and in silico study. Food and Chemical Toxicology. 2012;50:1124-33.
48. Chapin RE, et al. NTP-CERHR expert panel report on the reproductive and developmental toxicity of bisphenol A. Birth Defects Research Development and Reproductive Toxicology. 2008;83:157-395.
49. Newbold RR, et al. Long-term adverse effects of neonatal exposure to bisphenol A on the murine female reproductive tract. Reproductive Toxicology. 2007;24:253-8.

50. Newbold RR, et al. Prenatal exposure to bisphenol A at environmentally relevant doses adversely affects the murine female reproductive tract later in life. Environmental Health Perspectives. 2009;117:879-85.

51. Zama AM and Uzumcu M. Epigenetic effects of endocrine-disrupting chemicals on female reproduction: An ovarian perspective. Frontiers in Neuroendocrinology. 2010;31:420-39.

52. EPA. Integrated Risk Information System. Bisphenol A (CASRN 80-05-7). US Environmental Protection Agency. 1988.

53. EPA. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), Final Report. US Enviromental Protection Agency. 1998.

54. European Commission. Guidelines of the Scientific Committee on Food for the presentation of an application for safety assessment of a substance to be used in food contact materials prior to its authorisation. 2001.

55. EFSA. Scientific opinion on bisphenol A: evaluation of a study investigating its neurodevelopmental toxicity, review of recent scientific literature on its toxicity and advice on the Danish risk assessment of bisphenol A. EFSA J. 2010;8(9):1829.