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In Vivo Maternal and *In Vitro* BPA Exposure Effects on Hypothalamic Neurogenesis and Appetite Regulators

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Abstract

In utero exposure to the ubiquitous plasticizer, bisphenol A (BPA) is associated with offspring obesity. As food intake/appetite is one of the critical elements contributing to obesity, we determined the effects of *in vivo* maternal BPA and *in vitro* BPA exposure on newborn hypothalamic stem cells which form the arcuate nucleus appetite center. For *in vivo* studies, female rats received BPA prior to and during pregnancy via drinking water, and newborn offspring primary hypothalamic neuroprogenitor (NPCs) were obtained and cultured. For *in vitro* BPA exposure, primary hypothalamic NPCs from healthy newborns were utilized. In both cases, we studied the effects of BPA on NPC proliferation and differentiation, including putative signal and appetite factors. Maternal BPA increased hypothalamic NPC proliferation and differentiation in newborns, in conjunction with increased neuroproliferative (Hes1) and proneurogenic (Ngn3) protein expression. With NPC differentiation, BPA exposure increased appetite peptide and reduced satiety peptide expression. *In vitro* BPA-treated control NPCs showed results that were consistent with *in vivo* data (increase appetite vs satiety peptide expression) and further showed a shift towards neuronal versus glial fate as well as an increase in the epigenetic regulator lysine-specific histone demethylase1 (LSD1). These findings emphasize the vulnerability of stem-cell populations that are involved in life-long regulation of metabolic homeostasis to epigenetically-mediated endocrine disruption by BPA during early life.

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Ethical Approval on Animal Research: Studies were approved by the Animal Research Committee of the Los Angles Biomedical Research Institute at Harbor-UCLA Medical Center and were conducted in strict accordance with guidelines provided by the American Accreditation Association of Laboratory Care and the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and conform to the principles and regulations as described in the Editorial by Grundy (Grundy, 2015). All animals were treated humanely and with regard for alleviation of suffering. Virgin Sprague Dawley female rats (Charles River Laboratories, Hollister, CA) were housed in an animal facility with controlled 12/12 hour light/dark cycles, constant temperature and humidity conditions and *ad libitum* access to chow diet (Lab Diet 5001; Brentwood, Missouri).

Keywords

Neuroprogenitor cells; proliferation, differentiation; obesity; perinatal exposures; epigenetic

INTRODUCTION

Bisphenol A (BPA) is a monomer plasticizer used in the manufacture of common household goods including polycarbonate plastics (e.g. food and drink containers), paints and adhesives(Vandenberg et al., 2007). As an estrogen endocrine disrupter chemical, BPA has been associated with a range of adverse perinatal, childhood, and adult health outcomes (Rochester, 2013), including reproductive and developmental effects (Kim et al., 2011), neurogenesis (Kim et al., 2009), neurological behaviour (Palanza et al., 2008), and metabolic disease (Teppala et al., 2012). BPA exposure has been linked to childhood and adult obesity and likely contributes to the on-going obesity epidemic (Di Ciaula and Portincasa, 2017; Janesick and Blumberg, 2012). In rodents, maternal BPA exposure increases postnatal body weights and growth rates, with some studies showing greater susceptibility to BPA-increased adiposity in female as compared to male offspring (Richter et al., 2007; Rubin and Soto, 2009; Somm et al., 2009). Critically, fetal exposures to BPA at levels equivalent to, or below the established daily human safe-dose (50μg BPA/kg body weight/day) not only increase body weight and postnatal growth rate, but also alter body composition in later life (Alonso-Magdalena et al., 2006; Alonso-Magdalena et al., 2010; Richter et al., 2007; Rubin and Soto, 2009; vom Saal et al., 2012)

One of the critical determinants of energy balance include energy (calorie) intake (Hill et al., 2012). The arcuate nucleus (ARC) of the hypothalamus is the key regulator of appetite, containing both orexigenic (neuropeptide Y, NPY; agouti-related peptide, AgRP) and anorexigenic (pro-opiomelanocortin, POMC) neurons involved in central regulation of food intake (Blevins et al., 2002). Orexigenic and anorexigenic neurons develop before birth, in preparation for extra-uterine life, (Kagotani et al., 1989) however functional projections are established during the early postnatal period in rodents (Grove et al., 2001; Nilsson et al., 2005; Padilla et al., 2010; Walsh and Brawer, 1979). Studies including those by our laboratory have shown prenatal nutrition-mediated effects on ARC neurogenesis resulting in a shift from satiety to appetite neurons in association with offspring hyperphagia and obesity (Staples et al., 2017; Val-Laillet et al., 2017). We have further shown that hypothalamic neuroprogenitor cell (NPC) proliferation (self-renewal) and differentiation (generation of neurons/glial cells) are vulnerable to endocrine disruption, with potential long-term consequences for appetite regulation and energy balance (Desai et al., 2011a; Desai et al., 2011b). Notably, BPA has been shown to influence neurogenesis in humans (Preciados et al., 2016) and animal models (Kim et al., 2009). In mice, prenatal exposure to BPA increases neurogenesis and neuronal migration (Nakamura et al., 2006) resulting in altered brain structure (Nakamura et al., 2007) and function (Nakamura et al., 2012).

Neurogenesis is regulated, in part, by basic-helix-loop-helix (bHLH) genes including differentiation repressor genes (e.g., Hes1) that maintain the NPC population, and activator genes (e.g. Math3; Mash1; Neurogenin, Ngn), which accelerate neurogenesis and

differentiation (Kageyama et al., 2008; Masica et al., 1971; Ohtsuka et al., 2001). Maternal BPA up-regulates Math3 and Ngn2 in mouse embryos, (Nakamura et al., 2006) and accelerated neurogenesis due to BPA exposure may reduce the population of NPCs in fetal (e14.5) mice (Komada et al., 2012; Nakamura et al., 2006). However, the effects of perinatal BPA on rat hypothalamic NPC cell proliferation and differentiation have not been determined.

We studied the effects of maternal BPA exposure during pregnancy on cultured hypothalamic NPCs from 1 day old newborns and examined development of appetite/satiety neurons (Desai et al., 2014). To more fully explore the mechanisms of BPA-mediated effects, we then utilized established models of newborn rat primary hypothalamic NPCs (which ultimately form appetite/satiety neurons), exploring both proliferative (i.e., trophic) and differentiation effects of BPA (Desai et al., 2011a; Desai et al., 2011b). We further explored putative signal factors which explain, in part, NPC responses, and underlying epigenetic mechanisms mediated by BPA. Our results demonstrate marked effects of BPA on hypothalamic progenitor cell proliferation as well as differentiation. These findings emphasize the vulnerability of stem-cell populations that are involved in life-long regulation of metabolic homeostasis to endocrine disruption by BPA during early life.

MATERIALS AND METHODS

BPA Models

***In Vivo* Maternal BPA Exposure:** Studies were approved by the Animal Care Committee at the Los Angeles Biomedical Research Institute at Harbor-UCLA and were in accordance with the American Accreditation Association of Laboratory Care. All animals were treated humanely and with regard for alleviation of suffering. Virgin Sprague Dawley female rats (Charles River Laboratories, Hollister, CA) were housed in an animal facility with controlled 12/12 hour light/dark cycles, constant temperature and humidity conditions and *ad libitum* access to chow diet (Lab Diet 5001; Brentwood, Missouri). To avoid potential BPA contamination, polypropylene cages and purified water in glass bottles were utilized. Female rats were randomly assigned to Control (n=6) or BPA (n=6) group. Control rats had access to purified drinking water, whereas the BPA group received purified drinking water containing BPA (5mg/L; BPA Sigma-Aldrich, purity ≥ 99%, CAS no. 80-05-7) for two weeks prior to mating and throughout pregnancy (Table 1). Among studies administering BPA to pregnant rodents via drinking water, a concentration of 10 mg/l water (consumption of ~1.2 mg/kg BW/day) (Mendoza-Rodriguez et al., 2011) produced BPA tissue concentrations of 10-25 ng/g tissue (Kabuto et al., 2004; Nakajima et al., 2012) consistent with that of human samples (Schonfelder et al., 2002). A gavage dose five-fold higher (6 mg/kg BW/day) achieved a significant increase in maternal serum BPA concentration (Yoshida et al., 2004), whereas a water concentration of only 1 mg/l resulted in low maternal plasma free BPA levels (0.84 ng/ml) (Patisaul et al., 2012). Our dose was selected based upon our confirmation (pilot study) of maternal and newborn serum levels within the lower range of demonstrated human levels with normal BPA exposure.

Maternal blood prior to mating was obtained via tail bleed and newborn blood was collected in BPA-free tubes for BPA analysis. We did not obtain blood samples during pregnancy as

blood collection via tail vein is known to induce stress, resulting in fetal resorption (Weinstock, 2017). Further, maternal stress has been demonstrated to be an independent risk factor for offspring obesity and for impacting brain development (Hohwu et al., 2014; Moog et al., 2018; Mueller and Bale, 2006). Free (unconjugated) BPA levels were measured using GC/MS (NMS Labs, PA) with assay sensitivity of 0.25 ng/ml. Insufficient plasma volume from maternal and newborns necessitated pooling of samples and hence only mean values are reported.

Dams gave birth spontaneously and at 1 day of age, four males from one litter were sacrificed, hypothalamus dissected and samples pooled (representing N=1) for primary NPC culture studies (described below). A total of 6 litters of Control and 6 litters of BPA group were studied.

***In Vitro* BPA Exposure:** An additional four control litters were studied for *in vitro* effects of exogenous BPA on NPCs. From each litter, hypothalamus was dissected from 1 day old Control males (four pooled samples representing N=1) for cell culture studies (Table 1). Passage 2 NPCs cultured in complete medium were treated with DMSO (control) or BPA (1, 10, 20 μM) for 5 days. For NPC differentiation studies, passage 2 cells were treated with 10μM BPA (see below). The total number studied for NPC cultures was N=4 from 4 litters.

Primary Cultures

Hypothalamic NPC Cultures: NPCs were isolated and grown as neurospheres, as described previously in detail (Desai et al., 2011a). Briefly, hypothalamus was dissected in DMEM/F12 medium, cells dissociated by trypsin, centrifuged and cells seeded (~5×10^4 cells/ml) in complete medium [NeurobasalTM Medium containing 1% anti-anti (Invitrogen), 2% B27 (GIBCO, Cat# 17504-044), 20ng/ml FGF2 (Sigma), 20ng/ml EGF (Sigma), 1μg/ml heparin (Lylli), and 2.5μg/ml L-glutamine (Invitrogen)]. After 8 days in culture (passage 0), centrifuged neurospheres were dissociated into single-cell by trypsinization and reseeded (passage 1) in complete medium. For induction of differentiation, dissociated cells were re-suspended in differentiating medium (in absence of FGF2, EGF and heparin) and seeded in culture dishes pre-coated with 0.01% poly-L-lysine (Sigma). Cells in complete and differentiated medium were harvested and protein extracted for analysis as described under Western Blot.

Analysis

NPC Proliferation Assay: NPC (complete medium) proliferation was determined as previously reported (Desai et al., 2011a) using MTT colorimetric assay (Mosmann, 1983).

Western Blot: Protein was extracted and Western Blotting performed as described previously (Desai et al., 2008). For NPCs, protein expression analysis included NPC marker (Nestin, Sigma); neuroproliferative and (Hes1, 35 kDa, Santa Cruz), proneurogenic (Mash1, 30 kDa, Santa Cruz; Ngn3, 23 kDa, abcam) factors; markers of neuron (Tuj1, 50 kDa) and astrocyte (GFAP, 46 kDa, Dako), orexigenic (AgRP, 14 kDa, Santa Cruz; NPY) and anorexigenic (POMC, 30 kDa, Santa Cruz) neuropeptides, and; epigenetic regulators DNA

methyltransferase 3a (DNMT3a, 120 kDa, Santa Cruz) and lysine (K)-specific histone demethylase 1A (LSD1, 110 kDa, Cell Signaling).

Immunostaining: Staining of cultured NPC has been previously described (Desai et al., 2011a; Desai et al., 2011b). Briefly, disassociated neurosphere cells in complete or differentiating medium were fixed in 4% paraformaldehyde in PBS and stained with rabbit anti-nestin (Sigma), anti-Hes1(Santa Cruz) or anti-Ngn3 (abcam). Secondary antibodies were donkey anti-rabbit IgG-Alexa 488 or donkey anti-mouse IgG-Alexa 568.

Statistical Analyses:

In vivo responses between BPA and control offspring were compared by unpaired t-test. *In vitro* responses between BPA exposed and untreated (DMSO) Control cells were compared by unpaired t-test or analysis of variance (ANOVA) with Dunnett's post-hoc test, as appropriate. P values ≤ 0.05 were considered significant.

RESULTS

Maternal BPA Effects

Plasma BPA Levels: The average water consumption over the course of pregnancy was similar in BPA and Control dams (BPA=47.4±3.0 ml/day; Control = 46.4±3.7 ml/day). Prior to BPA administration, the pooled maternal plasma BPA value was 0.46 ng/ml. The amount of BPA consumed by dams via drinking water was approximately 500-900 μg/kg/day during pregnancy. Newborns of BPA dams had higher plasma BPA levels (0.62 ng/ml) as compared to undetectable levels in newborns of Control dams.

Maternal BPA Effects on Offspring Hypothalamic NPCs: At 1 day of age, neurospheres from BPA males cultured in complete media had increased NPC proliferation and increased expression of the NPC marker (Nestin). The bHLH proliferative factor Hes1 was increased in BPA-exposed as compared to Control NPCs (Figure 1). In differentiation media, BPA NPCs showed increased expression of the pro-differentiation neurogenic factor Ngn3 by both immunostaining and protein expression as compared to Controls (Figures 2A, B). Importantly, BPA NPCs exhibited enhanced differentiation toward appetite as compared to satiety neurons, as evident by increased protein expression of AgRP and decreased expression of POMC (Figure 2C).

In Vitro BPA Effects

NPCs: We also examined the effects of *in vitro* exposure to BPA on cultures from Control hypothalamic tissue. Control hypothalamic NPCs cultured in complete media with BPA showed dose-dependent increased NPC proliferation (Figure 3A), consistent with increased expression of the NPC marker Nestin and the proliferation bHLH factor Hes1 (Figure 3B), confirmed by double immunostaining (Figure 3C). In differentiation media, BPA promoted NPC differentiation toward increased neuronal (Tuj1) as compared to glial (GFAP) cell fate (Figure 4A), in conjunction with increased expression of the differentiation bHLH factors Mash1 and Ngn3. Consistent with the *in vivo* results (above), BPA enhanced differentiation

toward appetite as compared to satiety neurons, as evident by increased protein expression of AgRP/NPY and decreased expression of POMC (Figure 4B).

Epigenetic Factors: BPA treated NPCs in complete media demonstrated no change in DNMT3, but significantly increased LSD1 (Figure 5).

DISCUSSION

The effects of prenatal BPA exposure on offspring hypothalamic NPC proliferation and differentiation, and the potential underlying mechanism involving regulatory transcription factors have not been previously explored. The results of the present study suggest that BPA-induced dysregulation of hypothalamic NPC proliferation and differentiation may influence appetite regulation and contribute to obesity.

Measurable BPA levels are seen in adults and children, including breast milk (1.1 ng/ml), maternal (0.2- >10 ng/ml) and fetal/newborn serum (0.2-9.2 ng/ml). More importantly, the higher levels reported in amniotic fluid (8.3-8.7 ng/ml) and placental tissues (1.0-104.9 ng/ml) (Kosarac et al., 2012; Padmanabhan et al., 2008), suggest the continued exposure of the fetus to BPA throughout development. BPA levels are higher in infants and children than in adults (Welshons et al., 2006) and notably, are associated with increased adiposity (Harley et al., 2013; Rochester, 2013). Animal studies confirm the association of BPA with adiposity and note that it is low (≤500 μg/kg/day) rather than high dose (>5,000 μg/kg/day) of maternal BPA that is effective in promoting offspring weight gain (Angle et al., 2013; Somm et al., 2009). Increased male and female postnatal growth is seen at maternal BPA doses between 2.4-500 μg/kg/day (Richter et al., 2007; van Esterik et al., 2014; Wei et al., 2011) with sex-specific effects seen in food intake. Males though not females exhibit age-related increased food intake (Angle et al., 2013).

As BPA-mediated adiposity is dependent, in part, on enhanced food intake (suckling) (Dyer and Rosenfeld, 2011; Garza and Butte, 1990; Ojha et al., 2013), we investigated the effects of *in vivo* and *in vitro* BPA exposure on hypothalamic NPCs that ultimately produce appetite and satiety neurons (Miller and Gauthier, 2007; Sousa-Ferreira et al., 2011). Exposure to BPA *in vivo* and *in vitro* increased both the proliferation and differentiation of hypothalamic NPCs, consistent with previous studies of BPA-effects on rat embryonic neural stem cell cultures (Okada et al., 2008; Tiwari et al., 2014), and murine neurogenesis *in vivo* (Itoh et al., 2012; Kim et al., 2009; Komada et al., 2012; Nakamura et al., 2006). Specifically, maternal BPA exposure induced a marked trophic effect on the hypothalamic NPCs from 1 day old offspring, as indicated by increased NPC proliferation index and increased NPC protein expression of the NPC marker, Nestin. Further, BPA increased the protein expression of the bHLH proliferation transcription factor Hes1, which suppresses neuronal differentiation by inhibiting proneurogenic bHLH factors Mash1 and Ngn3 (Kageyama et al., 2008). In differentiating medium, BPA exposure increased Ngn3, indicating increased neuronal differentiation. More importantly, Mash1 and Ngn3 are required for development of POMC/NPY neurons (McNay et al., 2006; Pelling et al., 2011) and notably, BPA NPCs showed increased appetite (AgRP) versus satiety (POMC) peptide expression.

In vitro BPA exposure complemented the *in vivo* exposure findings and further demonstrated increased expression of Mash1 and Ngn3, indicating that there was increased neurogenesis as opposed to astrogliogenesis (increased Tuj1/GFAP ratio). These data are consistent with accelerated neurogenesis, consistent with BPA effects on murine neocortical and hippocampal structure (Jang et al., 2012; Komada et al., 2012; Kunz et al., 2011; Nakamura et al., 2007; Nakamura et al., 2006) synaptogenesis (Kagotani et al., 1989; Xu et al., 2013) and cerebellar granule neuron development (Mathisen et al., 2013). In addition, studies of embryonic zebrafish demonstrate that low dose BPA causes increased neurogenesis at birth (Kinch et al., 2015). Notably, endogenous neurodifferentiation factors may preferentially direct NPC differentiation towards neuronal (e.g., leptin) or astrocyte fate (e.g., insulin) (Desai et al., 2011a; Garza et al., 2008; Machida et al., 2012). Whether BPA exposure alters the relative expression of neuronal to glial cells *in vivo* (Okada et al., 2008) is unknown, but of concern. However, a recent study by MacKay et al (MacKay et al., 2017) demonstrates in vivo BPA effects on specific hypothalamic pathways involving melanocortin circuitry. Young adult offspring exposed to prenatal BPA exhibited a delayed postnatal leptin surge with leptin resistance, and showed a reduced density of POMC projections into the hypothalamic paraventricular nucleus (PVN). Notably daily injections of supplemental leptin in BPA exposed pups, rescued POMC projections into the PVN.

The mechanism underlying BPA-induced enhanced NPC proliferation and differentiation may involve epigenetic modifications (Bastos et al., 2013; Kundakovic and Champagne, 2011), particularly altered methylation of gene Hes1 (Lillycrop et al., 2015). Methylation and demethylation is catalyzed by enzymes DNMT (DNA methyltransferase) and LSD1 (lysine (K)-specific histone demethylase), respectively, both of which have been implicated in determining stem cell proliferation (self-renewal) and differentiation (Adamo et al., 2011; Wu et al., 2012). For example, Dnmt3a or Lsd1 knockout mice demonstrate impaired neuronal production coupled with increased astrogliogenesis and reduced NPC proliferation, respectively (Sun et al., 2010; Wu et al., 2012). Although we demonstrated no change in DNMT3a, the increased protein expression of LSD1 in response to BPA is consistent with an epigenetically-mediated shift toward neurogenesis. Previous studies show that perinatal BPA exposure alters brain DNMT3a which is region specific with increased mRNA levels of Dnmt3a seen in the cerebral cortex and no changes evident in the hippocampus (Kumar and Thakur, 2017) Dnmt1 and Lsd1 are highly interrelated and rely mechanistically on each other for normal chromatin function in vivo. Targeted deletion of the gene encoding Lsd1 in embryonic stem cells induces progressive loss of DNA methylation. This loss correlates with a decrease in DNMT1 protein, as a result of reduced Dnmt1 stability (Wang et al., 2009). However, it is unclear whether the effects of Lsd1 deficiency are mediated through an inability of Dnmt3a to catalyse 5mC, or via direct effects on the maintenance methyltransferase Dnmt (Rose and Klose, 2014).

Notably, LSD1 interacts with Notch pathway (Lopez et al., 2016), which is involved in neurogenesis and regulation of Hes1 expression (Imayoshi et al., 2010), and shown to specifically target the expression of bHLH gene *HEYL* (Hirano and Namihira, 2016a; Hirano and Namihira, 2016b). While the current data suggest BPA alters transcriptional regulation of genes involved in proliferation and differentiation by demethylation within

hypothalamic NPCs, further studies of site-specific epigenetic modification of specific genes are required to fully elucidate BPA-induced changes in neurogenesis.

Developing brain is more vulnerable to BPA due to its lipophilic chemical structure that allows it to easily cross the blood-placental and blood-brain barriers, impacting neurogenesis and thereby brain physiology. Although BPA mimics estradiol effects (Rubin, 2011), it may exert its action via differing pathways. Estradiol upregulates neurogenesis (Barker and Galea, 2008; Tanapat et al., 1999) and exerts its actions primarily through the genomic pathway involving nuclear estrogen receptors (Quaedackers et al., 2001). In contrast, BPA has higher affinity for membrane-bound G protein-coupled estrogen receptors (Thomas and Dong, 2006). Thus, BPA may act through both pathways in promoting NPC (Okada et al., 2008) proliferation. BPA interferes with the dimorphic development of the neuronal networks controlling brain functions (Delfosse et al., 2014; Wolstenholme et al., 2011) and alters dimorphic feeding behaviour (Liang et al., 2002; Negri-Cesi, 2015; Titolo et al., 2006). Specifically, estrogen can modulate the production of NPY and AgRP (Titolo et al., 2006) and mediate anorectic properties (Liang et al., 2002) by influencing POMC neurons in the ARC (Gao et al., 2007). The activity of this neuronal circuitry is gender specific, with females showing responsiveness to various anorectic inputs different from that of males (Mackay et al., 2013).

CONCLUSION

These data confirm that primary neuroprogenitor cells are vulnerable to endocrine disruption by BPA resulting in altered proliferation and differentiation, independent of systemic influences. Enhanced proliferation coupled with increased differentiation of NPCs to appetite as compared to satiety neurons indicate the potential for maternal/fetal BPA exposure to program an increased risk of offspring obesity (Ding et al., 2014; Miyawaki et al., 2007; Perreault et al., 2013; Somm et al., 2009). Of equal importance, the marked shift in NPC differentiation to neuronal versus glial fates may adversely impact cerebral development (e.g., cognition, behaviour) in regions beyond the appetite network. Future study that addresses long-term effect of altered neurogenesis and whether similar changes are evident in females should overcome the limitation of the present study

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Abbreviations

ARC	Arcuate nucleus
bHLH	Basic-helix-loop-helix

LSD1	Demethylase enzyme
NPY	Neuropeptide Y
POMC	Pro-opiomelanocortin
AgRP	Agouti-related peptide
BPA	Bisphenol A
Ngn	Neurogenin
NPCs	Neuroprogenitor cells

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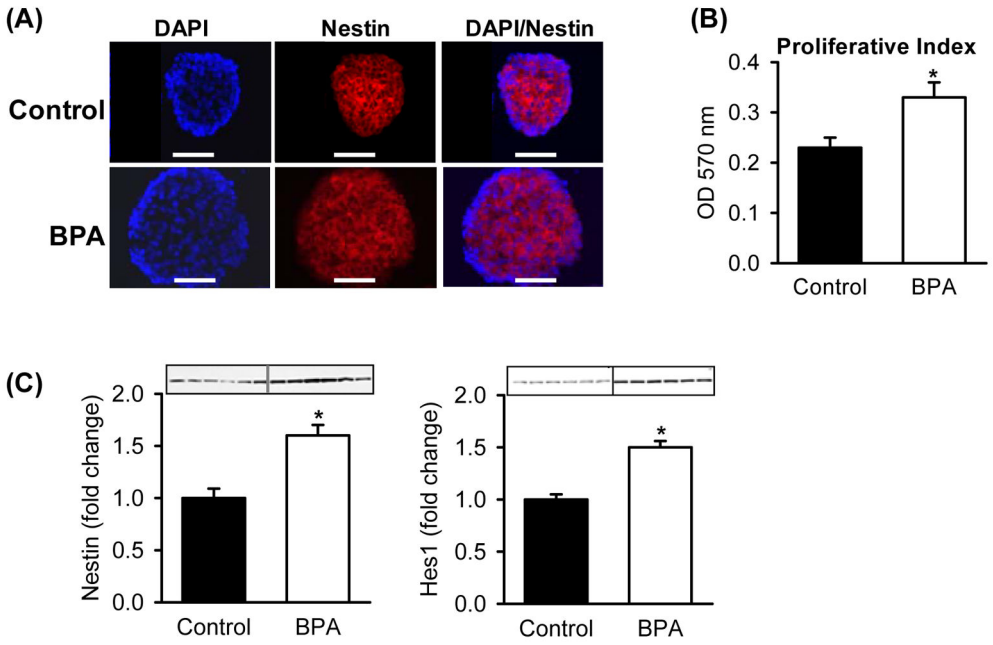


Figure 1: Maternal BPA Effects on Offspring NPC Proliferation

Hypothalamic NPCs from Control and BPA 1 day old male newborns were cultured in complete media. (A) Double immunostained images of (x40; scale bar = 50μm) of DAPI (blue, nuclear stain) and Nestin (red, NPC marker). (B) Proliferative index measured at 570 OD. (C) Protein expression of NPC marker (Nestin) and neuroproliferative factor (Hes1). Values are fold change (mean±SE) of n=6 of pooled hypothalami from each of 6 litters per group. * P< 0.05 BPA (□) vs. Control (■).

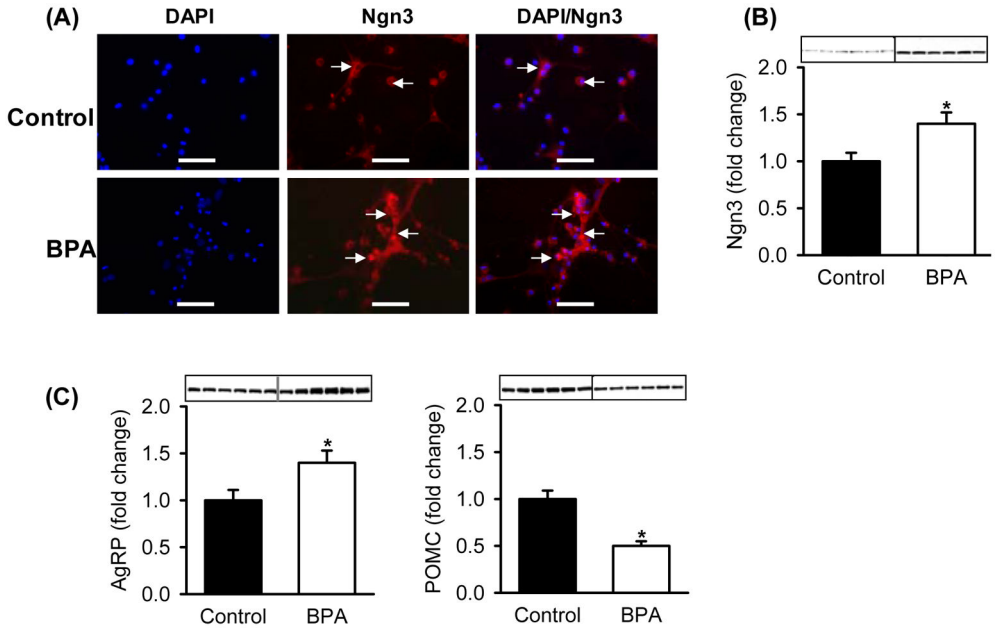


Figure 2: Maternal BPA Effects on Offspring NPC Differentiation and Neuropeptides

Hypothalamic NPCs from Control and BPA 1 day old male newborns were cultured in differentiation media. (A) Double immunostained images of (x40; scale bar = 50μm) of DAPI (blue, nuclear stain) and Ngn3 (red, proneurogenic factor). (B) Protein expression of Ngn3. (C) Protein expression of appetite (AgRP) and satiety (POMC) neuropeptides. Values are fold change (mean±SE) of n=6 of pooled hypothalami from each of 6 litters per group. * P< 0.05 BPA (□) vs. Control (■).

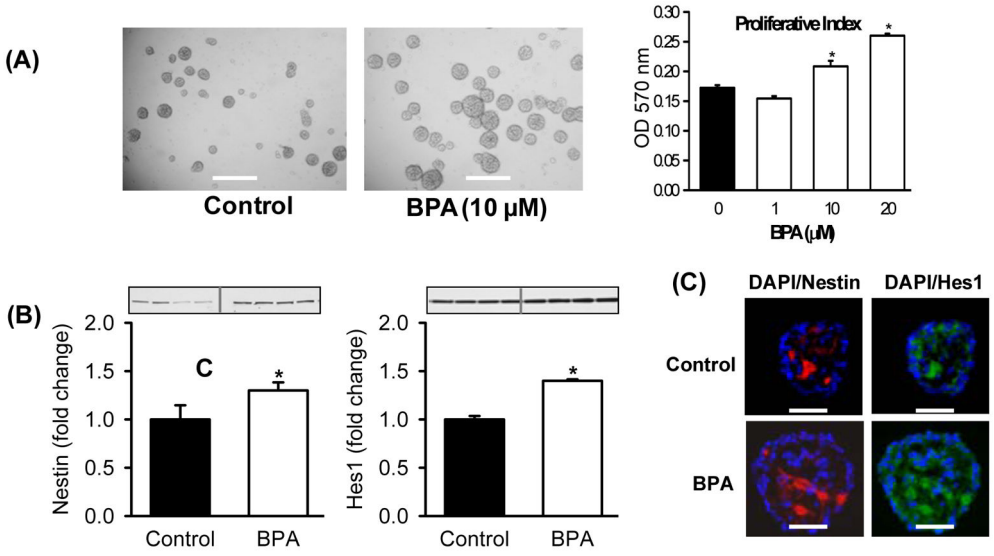


Figure 3: *In Vitro* BPA Effects on NPC Proliferation

Hypothalamic NPCs from 1 day old Control newborns were cultured in complete media and treated with DMSO (Control) or BPA (10 µM unless otherwise specified) for 5 days. (A) Images of NPCs (x20; scale bar = 50µm) and proliferative index measured at 570 OD of NPCs treated with BPA (1, 10, 20 µM). (B) Protein expression of Nestin (NPC marker) and bHLH proliferative (Hes1). (C) Immunostained images of (x40) Nestin (red), Hes1 (green) and DAPI (blue, nuclear stain). Values are fold change (mean±SE) of n=4 of pooled NPCs from each of 4 litters. * P< 0.05 BPA (□) vs. Control (■).

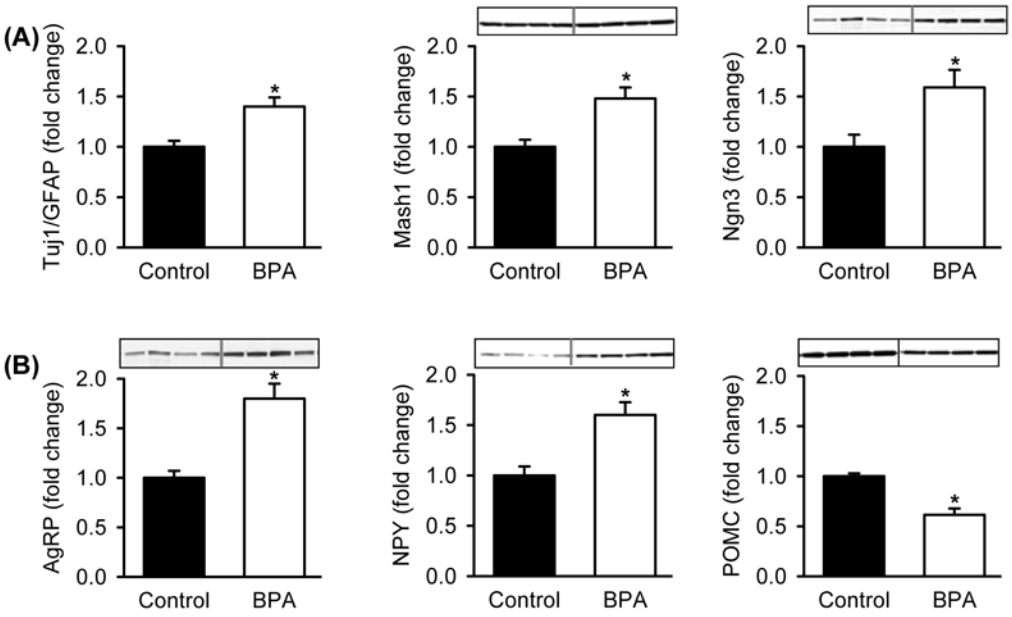


Figure 4: *In Vitro* BPA Effects on NPC Differentiation

Hypothalamic NPCs from 1 day old Control newborns were cultured in differentiation media and treated with DMSO (Control) or BPA (10 μM,) for 5 days. (A) Protein expression of bHLH proneurogenic factors (Mash1, Ngn3) and neuronal (Tuj1) and astrocyte (GFAP) markers. (B) Protein expression of appetite (AgRP, NPY) and satiety (POMC) neuropeptides .Values are fold change (mean±SE) of n=4 of pooled NPCs from each of 4 litters; * P< 0.05 BPA (□) vs. Control (■).

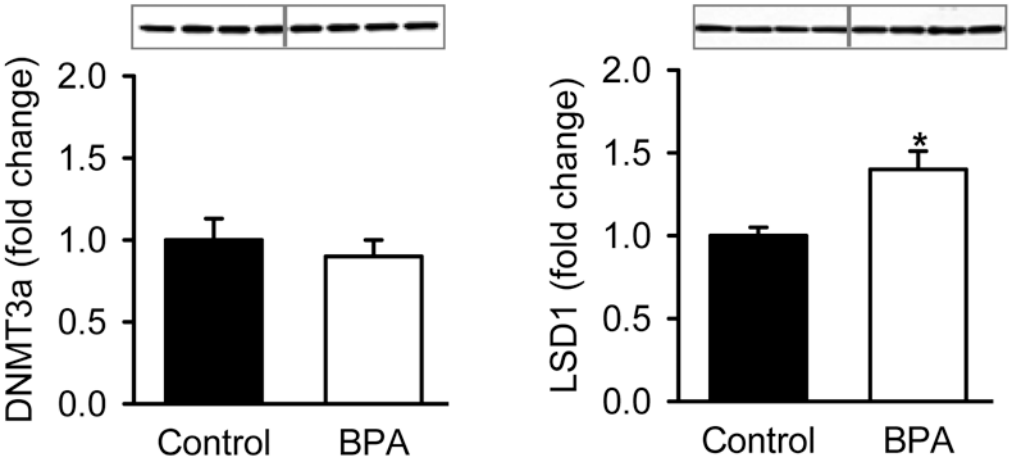
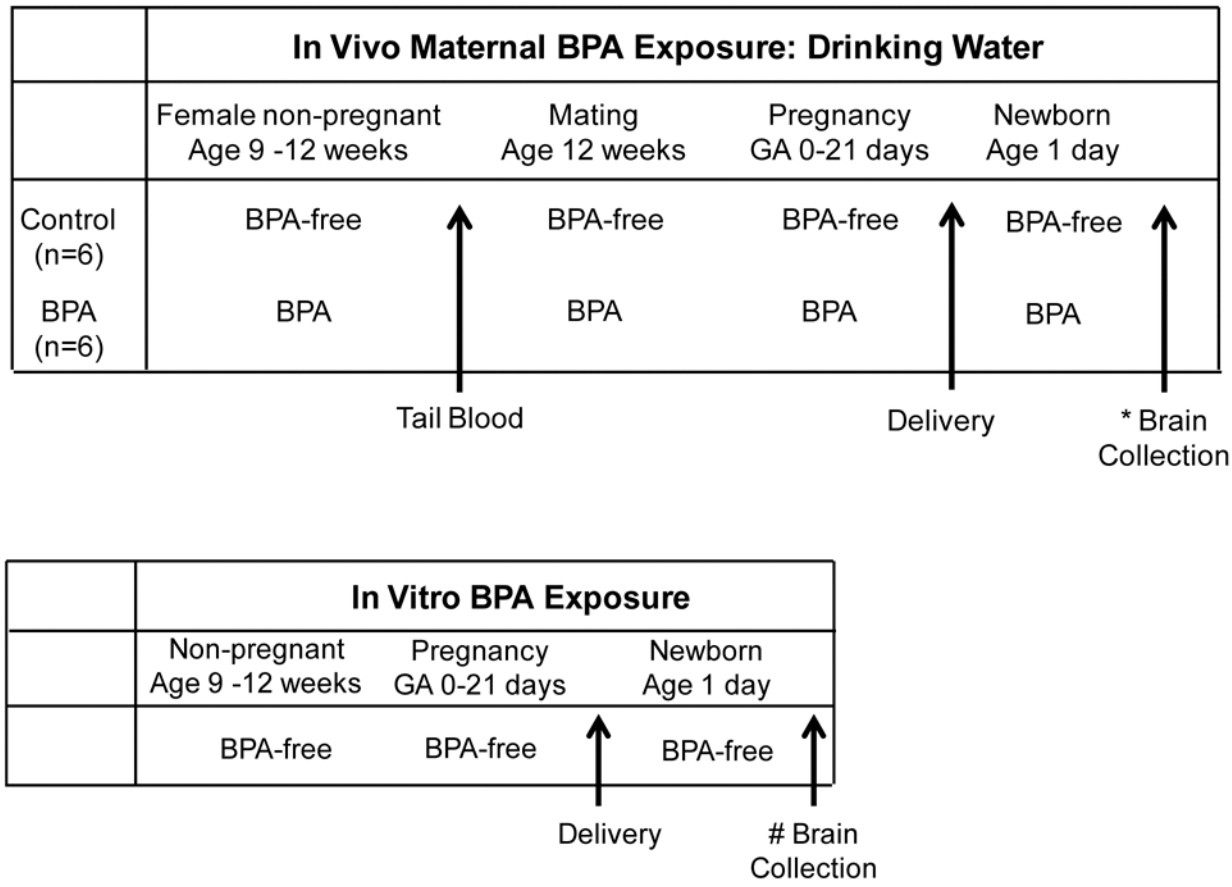


Figure 5: *In Vitro* BPA Effects on NPC Epigenetic Factors

Hypothalamic NPCs from 1 day old Control newborns were cultured in complete medium and treated with DMSO (Control) or BPA (10 μM,) for 5 days and protein expression of DNMT3a and LSD1 analyzed. Values are fold change (mean±SE) of of pooled cells n=4 from each of 4 litters; * P< 0.05 BPA (□) vs. Control (■).

Table 1:

Study Design of BPA Exposure



In Vivo Maternal BPA Exposure: Non-pregnant female rats at 9 weeks of age were allowed drinking water that was BPA-free (Control group) or contained BPA (BPA group). At 12 weeks of age, tail blood was obtained for BPA analysis and all females were mated and continued on same drinking water regimen throughout pregnancy. Brains were collected from their newborns for cultures of hypothalamic NPCs in BPA free media.

In Vitro BPA Exposure: Non-pregnant and pregnant dams had access to BPA-free drinking water. Brains were collected from their newborns for cultures of hypothalamic NPCs in media containing BPA.