Sex-specific *Esr2* mRNA expression in the rat hypothalamus and amygdala is altered by neonatal bisphenol A exposure

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Abstract

Perinatal life is a critical window for sexually dimorphic brain organization, and profoundly influenced by steroid hormones. Exposure to endocrine-disrupting compounds may disrupt this process, resulting in compromised reproductive physiology and behavior. To test the hypothesis that neonatal bisphenol A (BPA) exposure can alter sex-specific postnatal *Esr2* (*Erβ*) expression in brain regions fundamental to sociosexual behavior, we mapped *Esr2* mRNA levels in the principal nucleus of the bed nucleus of the stria terminalis (BNSTp), paraventricular nucleus (PVN), anterior portion of the medial amygdaloid nucleus (MeA), super optic nucleus, suprachiasmatic nucleus, and lateral habenula across postnatal days (PNDs) 0–19. Next, rat pups of both sexes were subcutaneously injected with 10 µg estradiol benzoate (EB), 50 µg/kg BPA (LBPA), or 50 mg/kg BPA (HBPA) over the first 3 days of life and *Esr2* levels were quantified in each region of interest (ROI) on PNDs 4 and 10. EB exposure decreased *Esr2* signal in most female ROIs and in the male PVN. In the BNSTp, *Esr2* expression decreased in LBPA males and HBPA females on PND 10, thereby reversing the sex difference in expression. In the PVN, *Esr2* mRNA levels were elevated in LBPA females, also resulting in a reversal of sexually dimorphic expression. In the MeA, BPA decreased *Esr2* expression on PND 4. Collectively, these data demonstrate that region- and sex-specific *Esr2* expression is vulnerable to neonatal BPA exposure in regions of the developing brain critical to sociosexual behavior in rat.

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Introduction

Sexually dimorphic brain organization, a process essential for establishing later in life – sex differences in neuroendocrine physiology and behavior, is profoundly influenced by endogenous steroid hormones during perinatal development. In rodents, brain masculinization is primarily induced by estrogens locally derived from circulating gonadal androgen

(Simerly 2002, McCarthy 2008, McCarthy *et al.* 2009). The absence of estrogens generally permits the ontogeny of female-typical brain development. Estrogen action is predominantly mediated by the two nuclear estrogen receptor (ER) subtypes: *ESR1* (*ERα*) (Greene *et al.* 1986) and *ESR2* (*ERβ*) (Kuiper *et al.* 1996). Administration of estrogens or aromatizable androgens to females, blockade of ERs in males, or exposure to endocrine-disrupting compounds (EDCs) during neonatal life disrupt the sexual differentiation process, resulting in compromised neuroendocrine pathways critical for mediating steroid negative feedback, gonadotropin release, energy homeostasis, and sociosexual behavior (Arai & Gorski 1968, Simerly 2002, Amateau *et al.* 2004, Gore 2008, Bader *et al.* 2011, Faulds *et al.* 2012, Patisaul *et al.* 2012). Although the relative role each *ESR* plays in the estrogen-dependent organization of sexually dimorphic neuroendocrine pathways remains unclear (Rissman 2008, Wilson & Westberry 2009, Fan *et al.* 2010, Handa *et al.* 2012), in the adult brain *ESR2* is thought to play a critical role in the mediation of estrogen-sensitive aspects of mood and social behaviors including

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aggression, anxiety, and sexual behavior (Handa *et al.* 2012). We have previously shown that *Esr1* and *Esr2* are dynamically expressed across neonatal development in subregions of the amygdala, as well as several anterior and mediobasal hypothalamic (MBH) nuclei in the rat brain. We have also shown that the sex-specific pattern of *ESR* expression, particularly *Esr2* expression, can be altered by exposure to the plastics component bisphenol A (BPA). In this study, we expand on that prior work and show that neonatal BPA exposure disrupts *Esr2* expression in additional regions of the amygdala (AMYG) and hypothalamus important for the coordination of sociosexual behavior and reproductive physiology. Collectively, with our prior work, these studies emphasize that BPA can alter *Esr2* expression in regions important for sex-specific behaviors.

BPA was first synthesized as a potential estrogenic compound in the 1930s (Dodds & Lawson 1936) and entered wide commercial use in the 1950s as a component of many household products such as polycarbonate plastics, epoxy resins, dental sealants, and thermal receipts (Vandenberg *et al.* 2007, Biedermann *et al.* 2010). BPA has been found in over 90% of all humans, with levels higher in children than in adults (Calafat *et al.* 2008), but the potential health impacts of this widespread, chronic, low level BPA exposure remain controversial. Growing evidence suggests that BPA is associated with adverse outcomes in reproductive (Howdeshell *et al.* 1999, Vandenberg *et al.* 2009, Beronius *et al.* 2010, Cabaton *et al.* 2011), cardiovascular (Pant *et al.* 2011), and metabolic (Groff 2010, Newbold 2010) health. In addition, animal and human data suggest that it may change many aspects of affective, cognitive, and reproductive behaviors (Negishi *et al.* 2004, Porrini *et al.* 2005, Rubin *et al.* 2006, Palanza *et al.* 2008, Cox *et al.* 2010, Patisaul *et al.* 2012, Rosenfeld 2012). Concern has been raised that early-life exposure to BPA may alter neural development and ultimately compromise neurobehavior (vom Saal *et al.* 2007, Chapin *et al.* 2008, NTP 2008, Palanza *et al.* 2008, Patisaul & Polston 2008, Report of Joint FAO/WHO Expert Meeting 2011, Wolstenholme *et al.* 2011, 2012, Rosenfeld 2012). Notably, in their 2008 evaluation of developmental and reproductive effects of BPA exposure, the National Toxicology Program (NTP) concluded that there was 'some concern for its effects on the brain and behavior' (Shelby 2008). In a 2010 statement, the FDA indicated similar concerns, although it continues to reaffirm its position that 'BPA is safe at the very low levels that occur in some foods' (<http://www.fda.gov/newsevents/publichealthfocus/ucm064437.htm>, updated March 2013).

The specific mechanisms by which early-life BPA exposure results in perturbed behavior remain unclear. Compared with estradiol, the binding affinity of BPA is relatively equivalent for *Esr1* and *Esr2* (Kuiper *et al.* 1998), but ~10 000- to 100 000-fold lower (Barkhem *et al.* 1998, Gould *et al.* 1998, Andersen *et al.* 1999, Blair *et al.* 2000). Thus, although BPA has long been considered to be weakly estrogenic, how it interacts with

molecular and cellular targets within the brain to alter estrogen-sensitive neural systems is not clearly established (vom Saal *et al.* 2007, Wolstenholme *et al.* 2011). We hypothesize that disruption of *ESR* expression during the process of brain sexual differentiation may be a mechanism by which BPA induces adverse effects on sex-specific behaviors such as anxiety and sociality.

Understanding how BPA and other EDCs may alter *ESR* expression during critical windows of brain development requires a detailed map of neonatal *ESR* distribution in both sexes. We previously reported that the expression of *Esr1* and *Esr2* mRNA is sexually dimorphic within numerous regions of the neonatal rat brain important for reproductive physiology and behavior (Cao & Patisaul 2011, 2013), including the preoptic area (POA), MBH (Cao & Patisaul 2011), and subregions of the AMYG (Cao & Patisaul 2013). We found that the degree to which neonatal expression is sexually dimorphic differs regionally and is often transient, with overall levels and the robustness of the sex difference changing with age. These observations support the hypothesis that the two primary isoforms of nuclear *ESR* may play different functional roles in the sexual differentiation process (Cao & Patisaul 2011, 2013). Moreover, we showed that neonatal BPA exposure decreased *Esr1* and, to an even greater extent, *Esr2* in POA (Cao *et al.* 2012), suggesting that *Esr2* is the more sensitive isoform to endocrine disruption. Furthering understanding of how BPA might disrupt *Esr2* in the developing hypothalamus is of particular interest because of the purported role *Esr2* has in the mediation of sociosexual behaviors.

For this study, we first mapped *Esr2* expression in the principal nucleus of the bed nucleus of the stria terminalis (BNSTp), paraventricular nucleus (PVN), anterior part of medial amygdaloid nucleus (MeA), super optic nucleus (SON), suprachiasmatic nucleus (SCN), and lateral habenula (LHb) across postnatal days (PNDs) 0–19. These region of interests (ROIs) were selected for several reasons. First, although there is an abundance of data regarding neural distribution of *Esr2* in adult rodents (Shughrue *et al.* 1997a, 1997b, Laflamme *et al.* 1998, Osterlund *et al.* 1998, Shughrue & Merchenthaler 2001, Mitra *et al.* 2003, Suzuki & Handa 2005, Chung *et al.* 2007), a detailed profile of *Esr2* mRNA expression from birth through weaning within these regions is incomplete. Second, most of these ROIs (BNSTp, PVN, MeA, SON, SCN, and LHb) express vasopressin (AVP), oxytocin (OT), and/or their receptors (Buijs *et al.* 1978, Brownstein 1980, De Vries *et al.* 1984, DeVries *et al.* 1985, Young & Gainer 2003, Caldwell *et al.* 2008). *Avp* expression of prenatal mouse brain was recently shown to be altered by BPA exposure at a dose considered to be human relevant (Wolstenholme *et al.* 2012), suggesting that *Esr2*-expressing nuclei within AVP/OT signaling pathways may be vulnerable to endocrine disruption by BPA. In adulthood, the PVN contains *Esr2*, but not *Esr1*, and *Esr2* is required to initiate estrogen-dependent OT and

AVP production in both sexes (Nomura *et al.* 2002, Patisaul *et al.* 2003). Although it is well established that early-life exposure to sex hormones affects the sexually dimorphic organization of the ROIs examined here, including OT and AVP pathways, few studies have specifically examined impacts on early-life *Esr2* expression (Perez *et al.* 2003).

Using tissues from a complementary, previously published study (Cao *et al.* 2012), the consequences of neonatal BPA exposure on *Esr2* expression in the developing brain was assessed in rat pups (both sexes) on PND 4 and PND 10 by administering vehicle, 10 µg estradiol benzoate (EB), 50 µg/kg BPA (LBPA), or 50 mg/kg BPA (HBPA) on PNDs 0–2 (the first 3 days of life) by s.c. injection. Although oral BPA administration is preferable when seeking to model human exposure and assess potential risk (Chapin *et al.* 2008, Li *et al.* 2008), because this study was mechanistic in nature and oral dosing to neonates can be stressful and laborious (Cao *et al.* 2013), s.c. injection was used. Collectively, the results demonstrate that the *Esr2* mRNA levels across the postnatal brain are dynamically altered. The data suggest that altered *Esr2* expression during the neonatal critical period may underlie reported disruptions of adult reproductive deficiencies and abrogated sex differences in sociosexual behavior across the lifespan. Future studies should explore the possibility that these effects might occur following exposures that better recapitulate human exposure conditions and doses.

Materials and methods

Animal care, neonatal exposure, and tissue collection

Tissues were obtained from two prior studies, the details of which are described elsewhere (Cao & Patisaul 2011, 2013, Cao *et al.* 2012). For these studies, 20 time pregnant Long-Evans (LE) rats were purchased from Charles River (Raleigh, NC, USA) and individually housed in a temperature, humidity and light-controlled room (23 °C, 50% average relative humidity and 14 h light:10 h darkness cycle; lights on at 0700 h) at the Biological Resource Facility of North Carolina State University (NCSU), according to the applicable portions of the Animal Welfare Act and the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. The procedures were approved by the Institutional Animal Care and Use Committee of NCSU. Both dams and rats were housed in thoroughly washed polysulfone (BPA-free) caging and fed with a semipurified, phytoestrogen-free diet *ad libitum* (AIN-93G, Test Diet, Richmond, IN, USA) to minimize exposure to exogenous BPA and other EDCs (Brown & Setchell 2001, Degen *et al.* 2002, Patisaul 2005, Thigpen *et al.* 2007, 2013).

Experiment 1: ontogeny of *Esr2* expression across male and female postnatal development

Pups were obtained as described previously (Cao & Patisaul 2011, 2013). Briefly, female and male pups ($n=5-7$ per sex,

per group) were killed by rapid decapitation on postnatal days 0 (PND 0 = the day of birth and defined as within 12 h after littering took place), 2, 4, 7, and 19. The whole head from the PND 0, 2, and 4 animals, and the brains from PND 7 and 19 animals, were rapidly frozen on powdered dry ice and stored at -80°C until cryosectioning. To minimize potential litter effects, no more than two pups of each sex from the same litter were collected at each time point; thus pups of the same sex are from at least three different litters.

Experiment 2: impact of neonatal BPA exposure on postnatal *Esr2* expression

As described previously, pups were obtained from 13 LE dams (Cao *et al.* 2012) and BPA was injected s.c. daily from PND 0 through PND 2 (a critical period that approximates the late-second and early-third trimesters of human brain development, Grumbach (2002)). Litter sizes ranged from 9 to 17 pups and were not standardized for size or sex ratio to minimize disruption of maternal care. Males and females ($n=6-9$ per sex per group) were randomly assigned to one of four groups: vehicle, EB (10 µg, Sigma), low-dose BPA (50 µg/kg bw; LBPA, Sigma), or high-dose BPA (50 mg/kg bw; HBPA). All pups within the litter were administered the same compound to prevent cross-contamination (3 l each for vehicle, EB, and LBPA, 4 l for HBPA), and each experimental group contained pups from at least 3 l to minimize potential litter effects. All compounds were first dissolved in 100% ethanol (EtOH, PharmaCo, Austin, TX, USA), and then sesame oil (Sigma) at a ratio of 10% EtOH and 90% oil (Patisaul *et al.* 2006). The vehicle was a mixture of 10% EtOH and sesame oil. The low dose is the oral reference dose considered 'safe' for human exposure, and the high dose is equivalent to the oral lowest observed adverse effect level (LOAEL; FAO/WHO 2011). Because these experiments were primarily mechanistic in nature, and assessing potential human risk was not a primary goal, injection was used for logistical reasons and to ensure consistent exposure across individual animals. Although injection may result in a higher internal dose than oral exposure (Doerge *et al.* 2010), at least one study has shown that this difference is not significant in neonatal mice (Taylor *et al.* 2008). EB was used as a positive control at a dose sufficient to induce complete masculinization of the hypothalamus and to prevent the onset of regular estrous cycles (Aihara & Hayashi 1989, Nagao *et al.* 1999). Pups were killed by rapid decapitation on PNDs 4 and 10, and the heads were rapidly frozen on powdered dry ice and stored at -80°C until cryosectioning.

In situ hybridization histochemistry

Brains were cryosectioned (Leica CM1900, Nussloch, Germany) into three serial sets of coronal sections (12 µm sections for Experiment 1 and 18 µm sections for Experiment 2), as described previously (Cao & Patisaul 2011, 2013, Cao *et al.* 2012), mounted onto Superfrost plus slides (Fisher Scientific, Pittsburgh, PA, USA), and stored at -80°C until *in situ* hybridization histochemistry (ISHH) processing. For each experiment, one set of sections containing all the ROIs from every animal (both sexes and all time points) were processed and analyzed

simultaneously as a large batch to eliminate batch effects. Thus two large batches of ISHH were performed: one for the mapping experiment (Experiment 1) and one for the BPA exposure experiment (Experiment 2). The templates for the antisense probes of *Esr2* were 501-bp cDNA fragments and cloned into the PCRII-TOPO vector. Details regarding probe specificity, synthesis and purification, and the ISHH procedure are available elsewhere (Cao & Patisaul 2011, 2013, Cao *et al.* 2012). Dried slides were exposed to Kodak Biomax MR X-ray film (Eastman Kodak) for 13 days for Experiment 1 and for 17 days for Experiment 2, along with an autoradiographic ^{14}C microscale (Amersham Life Sciences) to generate the optical density curve. The autoradiographic films were developed using a Konica SRX-101A processor (Konica Corporation, Tokyo, Japan). To confirm the results obtained from the autoradiograms, the slides were dipped in NTB3 emulsion (Kodak), kept at 4 °C for 73 days, and then developed in Dektol developer and Kodak fixant (Kodak) according to the user manual. Then all slides were counter-stained with Mayer's hematoxylin (Sigma), as described previously (Cao & Patisaul 2011, Cao *et al.* 2012), to visualize cell-specific silver grain clusters.

Landmark identification and image analysis

The MCID Core Image software program (InterFocus Imaging Ltd, Cambridge, Cambs, UK) was used to quantify *Esr2* mRNA signals, as described previously (Cao & Patisaul 2011, 2013, Cao *et al.* 2012). ROIs included BNSTp, PVN, MeA, SON, SCN, and LHb. An in-house library of Nissl stained sections was used to identify each ROI across neonatal development (Fig. 1A, B, C and D left panels, encircled with a solid line), and a rat brain atlas (Paxinos & Watson 2007) was referenced to confirm the location and borders of each ROI (Fig. 1A, B, C and D right panels; ROIs shaded light gray). The area selected (sampling template) for film quantification is encircled in the left panels of Fig. 1 with a dashed line. The size of this quantification area increased across age groups (to account for growth) but was standardized for all animals at that age.

All quantification was conducted by investigators blinded to the exposure groups. For each brain area, ROI and background levels were measured unilaterally from four anatomically matched sections. The resulting values for each brain section after background subtraction were then averaged to obtain a representative measurement (for that ROI) for each animal. Optical densities were converted to nCi/g tissue equivalents using a 'best fit' curve (5th degree polynomial) generated from the autoradiographic ^{14}C microscales. In all cases, the signal was within the limits of the curve. The measurements for the BNSTp and PVN were completed by two investigators blinded to the exposure groups. There was a high degree of concordance between the two data sets (Pearson's coefficient >0.98), thus the results were averaged to obtain the final values for those two regions. MeA, SON, and Pe measurements were made by one investigator. Within the SCN, the signal from PND 0 to 19 was so weak, that meaningful quantification was not possible. For other groups/ages in which no data was reported, either no signal was present or there were not enough quality sections per sample available to reach the minimum goal of three samples per group per age per sex.

Figure 1 Representative NISSL-stained sections from PND 2 rats depicting each brain region of interest (ROI, circled with a solid black line and indicated by the solid black arrows) and the corresponding sampling template created to define the sampling area (circled with a dashed black line and indicated by the open arrows) for autoradiographic quantification (A, B, C and D, left panel). A sampling template was created for each age according to the size and position of each brain area and then used for all sections, regardless of sex, within that age group. Representative sections containing a midlevel section of the BNSTp (A), PVN (B and C), SON (A and B), MeA (B and C), and LHb (D) were obtained from animals in our existing colony and used, along with a standard rat brain atlas (Paxinos & Watson 2007), to identify the ROI landmarks and anatomical borders for these studies. All depicted sections are matched with their corresponding coronal plates with the ROI shaded in gray; Bregma -0.84 mm for the BNST and SON, -1.56 mm for the PVN, SON and MeA, -1.92 mm for the PVN and MeA, and -3.12 mm for the LHb (A, B, C, D, right panel). For abbreviations, see list. Scale bar is 500 μm in the left panels of A, B, C and D.

Statistical analysis

For Experiment 1, all data was first analyzed by two-way ANOVA with sex and age as factors. For Experiment 2, data were analyzed within each age with sex and exposure group as factors. Significant main effects and/or interactions were followed by appropriate one-way ANOVA. And the Dunnett's Multiple Comparison *post hoc* test was used to compare each age group with PND 0 in Experiment 1, or to compare each exposure group with the vehicle control within in Experiment 2. For Experiment 2, sex differences at each age were identified by *t*-tests. All analyses were two-tailed and results were considered significant when $P \leq 0.05$ within each ROI.

Results

All results from both experiments are summarized in Tables 1 and 2, including the impact of BPA exposure on sexually dimorphic expression. Because *Esr2* signal was observed to be robust in the caudal portion of periventricular hypothalamic nucleus (Pe) on PND 19, it was subsequently quantified and included in the analysis for both experiments. Levels in the SCN were detectable at all ages but too low to be quantified.

Experiment 1: ontogeny of *Esr2* expression across male and female postnatal development

Esr2 mRNA was detected in the Pe, BNSTp, PVN, MeA, SCN, SON and LHb (Figs 2, 3 and 4). Consistent with what we have previously observed, *Esr2* signal was more robust in the BNSTp and PVN than all other regions examined (Figs 2, 3 and 4). *Esr2* mRNA levels were observable but too low to be quantified in the SCN (not shown). *Esr2* expression was observed in PND 0 LHb in both sexes, but it decreased with age to the limit of detection, and was thus not quantifiable. For comparison, *Esr1* expression was assessed in a companion set of sections labeled previously (Cao & Patisaul 2013) and no *Esr1* signal was observed (Fig. 4E, F). Thus *Esr* expression in the neonatal LHb appears to be *Esr2* only. Quantification across the entire postnatal period was only possible for the BNSTp and PVN (Fig. 2). Because signal was too weak to quantify in younger animals, *Esr2* expression was only quantified on PND 19 in the Pe (Fig. 3A) and MeA (Fig. 3B), and on PNDs 7 and 19 in the SON (Fig. 3C).

BNSTp

Quantification was mainly confined to BNSTp, located in the medial posterior division (Fig. 2A). BNSTp *Esr2* signal was expressed in both temporal- and sex-specific patterns (Fig. 2 and Table 1). Two-way ANOVA revealed a significant main effect of age ($F(4, 47)=20.93$, $P \leq 0.0001$), sex ($F(1, 47)=9.174$, $P \leq 0.004$) as well as a significant interaction ($F(4, 49)=5.744$, $P \leq 0.0008$). In females ($F(4, 24)=21.64$, $P \leq 0.0001$), expression was high on PND 0 but rapidly decreased with age and remained low from PNDs 2–19 ($P \leq 0.01$). In males, there was also a main effect of age ($F(4, 23)=10.76$, $P \leq 0.0001$) with levels peaking on PND 2, then significantly lower on PNDs 4–19. Sexually dimorphic expression of *Esr2* was found only on PND 2 (Fig. 2A and B) with higher levels in males ($P \leq 0.05$).

PVN

Esr2 mRNA level was robust in the PVN on all days examined (Fig. 2C and D). A main effect of age ($F(4, 45)=19.19$, $P \leq 0.0001$) was revealed by two-way ANOVA, but no effect of sex. Compared with same sex PND 0 animals, *Esr2* signal was significantly increased on PND 7 in males ($P \leq 0.05$), and in both sexes ($P \leq 0.01$) on PND 19 (Fig. 2C and D). Expression levels were compared between sexes at each age, but only found to be sexually dimorphic on PND 7, with higher levels in males (Fig. 2C and D and Table 1).

Pe, MeA, and SON

We previously observed robust *Esr2* expression in the rostral portion of the Pe on PND 19 (Cao & Patisaul 2011). Similarly, in this study, *Esr2* expression was observed to be present in the caudal portion of the PND 19 Pe (Fig. 3A), and was therefore quantified. Expression was sexually dimorphic ($P \leq 0.001$) with levels higher in PND 19 females than in males. *Esr2* expression was also detected in the MeA (Fig. 3B) but quantifiable only on PND 19 and not found to be sexually dimorphic. In the SON, signal was visible as early as on PND 0 (Fig. 3C) but quantifiable only on PNDs 7 and 19. Levels were higher on PND 19 compared with PND 7 and only a trend for sexually dimorphic expression ($P=0.07$) was found, with higher levels in females (Fig. 3C). Qualitative assessment of silver grain deposition on the emulsion-dipped slides confirmed that the sex difference was appreciable but not large.

Table 1 *Esr2* mRNA expression in postnatal rat brain without neonatal BPA exposure.

	PND 0	PND 2	PND 4	PND 7	PND 19
BNSTp	F=M	F<M	F=M	F=M	F=M
PVN	F=M	F=M	F=M	F<M	F=M
MeA	NA	NA	F=M	NA	F=M
SON	NA	NA	F<M	F=M	F>M*
Pe	NA	NA	NA	NA	F>M

Table 2 *Esr2* mRNA expression in postnatal (PND 0 to PND 2) rat brain with neonatal BPA exposure.

		Vehicle	EB	LBPA	HBPA
BNSTp	PND 4	F=M	F=M	F=M	F=M
	PND 10	F=M	↓F=M	F>M↓	↓F<M
PVN	PND 4	F=M	↓F=M↓	F=M	F=M↓
	PND 10	F<M	↓F=M↓	F>M↓	F=M
MeA	PND 4	F=M	↓F=M	↓F=M	↓F=M
	PND 10	F=M	F=M	NA	F=M↓
SON	PND 4	F<M	F=M	F=M↓	F=M↓
	PND 10	F=M	F=M	F=M	↓F=M↓

‘↓’ and ‘↓↓’ represent signal significantly decreased compared with vehicle control or PND 0 female and male respectively. * $P=0.07$ from *t*-test between female and male on PND 19. NA, analysis is not available.

LHb

In the LHb, *Esr2* mRNA was visible from PNDs 0–19 in both sexes on both the autoradiograms and the emulsion-dipped slides (Fig. 4). Comparison with adjacent sections labeled for *Esr1* confirmed that no cross reactivity occurred and that, although weak, the signal is exclusively *Esr2* and not *Esr1* (Fig. 4A, B, C and D) until PND 19. In a few PND 19 animals, a very weak signal for *Esr1* was observed on the autoradiograms and confirmed by silver grain deposition (Fig. 4E and F). Signal was insufficient for quantification but qualitatively it appears that there may be a sex difference in *Esr2* expression during PNDs 0–4 but not after.

Experiment 2: impact of neonatal BPA exposure on postnatal *Esr2* expression

For Experiment 2, *Esr2* signal was quantified on PNDs 4 and 10 in the BNSTp, PVN, SON, and MeA (Figs 5, 6 and 7). Signal was not robust enough to quantify the expression in the SCN or LHb.

BNSTp

In the PND 4 animals, two-way ANOVA did not reveal a significant effect of sex, exposure group, or a significant interaction for *Esr2* expression (Fig. 5 and Table 1). On PND 10, two-way ANOVA revealed a significant effect of exposure group ($F(3, 35)=29.15$, $P\leq 0.001$) and an interaction with sex ($F(3, 35)=19.22$, $P\leq 0.01$). One-way ANOVA within females revealed an overall effect of exposure ($F(3, 20)=11.71$, $P\leq 0.0001$), with both EB ($P\leq 0.05$) and HBPA ($P\leq 0.01$) significantly decreasing *Esr2* expression (Fig. 5C and D). In the PND 10 males, the main effect of exposure did not quite reach statistical significance ($F(3, 15)=3.169$, $P=0.055$), but the Dunnett's *post hoc* test revealed that *Esr2* expression was lower in the LBPA exposure group compared with the same-sex, unexposed controls ($P\leq 0.05$).

PVN

Two-way ANOVA indicated a significant effect of exposure ($F(3, 40)=8.12$, $P\leq 0.0002$) on PND 4 (Fig. 6), but no effect of sex or a significant interaction.

EB significantly decreased *Esr2* signal in both sexes ($P\leq 0.05$), while HBPA decreased *Esr2* signal only in males (Fig. 6A and B). On PND 10, two-way ANOVA revealed a significant effect of exposure ($F(3, 40)=8.087$, $P\leq 0.0002$) and a significant interaction with sex ($F(3, 40)=4.742$, $P\leq 0.0064$). As expected, on PND 10 a sex difference in *Esr2* expression was observed in the vehicle controls, with higher levels in males (Fig. 6C and D). EB exposure decreased *Esr2* mRNA expression in both sexes, thereby eliminating the sex difference. In the LBPA group, expression was significantly lower in males ($P\leq 0.05$) and higher in females ($P\leq 0.044$), compared with their same-sex controls, which reversed the sex difference in expression ($P\leq 0.05$). Expression in the HBPA group was not significantly altered in either sex compared with their control conspecifics, but altered enough such that the sex difference in expression was lost (Fig. 6D).

MeA

Esr2 expression was not sexually dimorphic on PND 4 or 10 (Table 1 and Fig. 7A, E, F and G). In the PND 4 animals, two-way ANOVA revealed a significant effect of exposure ($F(3, 45)=6.766$, $P\leq 0.0007$) and sex ($F(1, 45)=4.971$, $P\leq 0.03$). EB ($P\leq 0.01$), LBPA ($P\leq 0.01$), and HBPA ($P\leq 0.05$) all significantly decreased *Esr2* mRNA levels in females (Fig. 7A and E), which collectively accounted for the main effect of sex. On PND 10, only one sample from the male LBPA exposure group was available, so the data are presented for qualitative assessment only. One-way ANOVA indicated no exposure effect in females (ANOVA not performed in males). In males, a *t*-test revealed that *Esr2* signal was significantly lower in the HBPA males ($P\leq 0.05$) compared with the vehicle controls.

SON

On PND 4, *Esr2* expression in the SON was sexually dimorphic in the vehicle controls, with higher levels in males ($P\leq 0.05$). Two-way ANOVA identified a significant effect of exposure ($F(3, 42)=4.642$, $P\leq 0.007$) and a significant interaction with sex ($F(3, 42)=3.265$, $P\leq 0.03$). Both doses of BPA, but not EB, significantly decreased *Esr2* mRNA levels in males ($F(3, 22)=5.652$,

Figure 2 Autoradiographic images showing *Esr2* mRNA signal in the postnatal rat BNSTp (A) and PVN (C) of both sexes (females on left side, males on the right in each panel). Within the BNSTp, labeling was robust at birth and declined by PND 2 in females and PND 4 in males, and remained low PND 19 (A and B). In PVN, *Esr2* mRNA signal was high on the day of birth and unchanged through PND 4 (C and D). On PND 7, *Esr2* signal was significantly increased in males only, and thus showed a sexually dimorphic expression pattern. By PND 19, *Esr2* mRNA levels were equivalent in both sexes (C and D). Significant differences in expression compared with PND 0 levels are represented by $**P \leq 0.01$ for the females, and $^{\#}P \leq 0.05$ and $^{\#\#}P \leq 0.01$ for the males. Sex differences in expression at each age are represented by $^{\&}P \leq 0.05$. The sample size for each group is presented in the graphs, and the bar in graphs represents mean $\pm$ s.e.m. For abbreviations, see list. Scale bar is 1000 μ m for all images in A and C.

Figure 3 Autoradiographs depicting *Esr2* mRNA labeling in the Pe (A), MeA (B), and SON (C) of both sexes (females in the left panel, males in the right panel). In Pe, *Esr2* mRNA signal was higher in females than in males on PND 19 (A) but too weak to quantify at earlier ages. Similarly, *Esr2* signal could only be quantified in the PND 19 MeA, and no sex difference was observed (B). *Esr2* signal in SON was relatively weak before PND 7 (C), but increased from PND 7 to PND 19 in both sexes with a marginal ($P=0.07$) sex difference emerging on PND 19 (C). Silver grain deposition on the emulsion-dipped slides confirmed the presence of distinct *Esr2* signal in the SON (bottom left panel in C). Significant differences in gene expression are represented by *** $P \leq 0.001$ for the females and ### $P \leq 0.001$ for the males compared with the levels observed for PND 7. Significant sex differences are represented by &&& $P \leq 0.001$. The graphs depict mean $\pm$ s.e.m. and the sample size is provided for each age. For abbreviations, see list. Scale bar is labeled in each panel.

LHb-*Esr1* and *Esr2*

Figure 4 Representative autoradiographic images, and silver grain deposition of *Esr2* (right panel of A, B, C and E) and *Esr1* (left panel of A, B, C and E) signal in both sexes on PND 0, 4, and 19 demonstrating that the *Esr* expression in this region is exclusively the *Esr2* subtype. On the day of birth, *Esr2* signal was robust in both females (A, top right panel) and males (A, bottom right panel), no *Esr1* mRNA labeling was appreciable in either sex (left panel of A). Silver grain deposition confirmed the film results in both females (B, left for *Esr1* and right for *Esr2* on top) and males (B, left for *Esr1* and right for *Esr2* on bottom). Qualitatively, *Esr2* mRNA signal appeared to decrease with age in both sexes (right panels of C and E), and silver grain deposition (D and F) suggested decreased number of cells containing signal and decreased expression levels within labeled cells. *Esr1* mRNA signal was not observed on either the films or the emulsion-dipped slides from PND 0 to 7 (left panel of A, B, C, and E, D and bottom panel of F) in sequential sections, from the same animals, used to label *Esr2*. By PND 19, a very weak *Esr1* signal was observed on the autoradiographs in a subset of animals, and silver grain deposition confirmed it with only a small number of silver grains clusters appreciable (top panel of F). Signal strength was insufficient to quantify either *Esr1* or *Esr2*. Arrows indicate silver grain clusters. The scale bar is labeled in each panel.

$P \leq 0.005$), but the sex difference in expression was lost in all three groups (Fig. 7A, B and Table 1). On PND 10, two-way ANOVA only indicated an effect of exposure ($F(3, 38) = 4.746$, $P \leq 0.007$). *Esr2* signal was significantly lower compared with same sex conspecifics in the HBPA group ($P \leq 0.05$, Fig. 7C and D).

Discussion

These data are significant in that they i) constitute the most detailed mapping of *Esr2* expression in the BNSTp, PVN, SON, MeA, SCN, and LHb during the first 2 weeks of life, and ii) show that neonatal BPA exposure can suppress *Esr2* expression in sexually dimorphic brain regions fundamental to sociosexual behavior. Across postnatal development, *Esr2* expression was region and sex-specific, and significantly changed with age.

Accordingly, effects of neonatal BPA exposure were age, region, and sex-specific. These studies are the first to show that neonatal BPA exposure can perturb *Esr2* expression in the BNSTp, PVN, MeA, and SON. In all cases except the PND 10 female PVN, expression was reduced by BPA, and EB-related effects were directionally similar. These observations are consistent with our prior observations comparing the region-specific impact of BPA and exogenous estrogen on *Esr* expression in limbic subnuclei (Cao *et al.* 2012, 2013), and emphasize that BPA may not be simply acting as an estrogen mimic in the brain. Diminished *Esr* expression by BPA presumably results in reduced regional sensitivity to endogenous estrogen, thereby altering estrogen-dependent neural organization. Although the functional significance of disrupted postnatal *Esr* expression remains to be fully established, altered postnatal *Esr*

Figure 5 Representative autoradiographs of *Esr2* signal in the BNSTp on PND 4 (A) and PND 10 (C) after vehicle (OIL), EB, LBPA, and HBPA exposure (from left to right in both A and C). Optical density analysis of *Esr2* expression in the PND 4 (B) and the PND 10 (D) BNSTp showed that *Esr2* expression was significantly decreased in females on PND 4 and 10 by neonatal EB exposure (B and D, left panels). BPA had no effect in either sex on PND 4 (C) and on PND 10, reduced *Esr2* mRNA levels were observed in LBPA males, but not in LBPA females, while the reverse was true in HBPA animals (D). Significant differences in expression compared with vehicle are represented by * $P \leq 0.05$, ** $P \leq 0.01$ for the females, and # $P \leq 0.05$ for the males. The graphs depict mean $\pm$ S.E.M. and the sample size is provided at the bottom (3V, third ventricle; scale bar = 1000 μ m).

Figure 6 Autoradiographs depicting *Esr2* signal in the PVN on PND 4 (A) and on PND 10 (C) after neonatal vehicle (OIL), EB, LBPA, and HBPA exposure (from left to right in both A and C). Reduced *Esr2* expression following neonatal EB exposure was observed in both sexes on PND 4 (B) and PND 10 (D), which eliminated the expected sex difference in expression on PND 10. BPA only significantly impacted *Esr2* expression in males. On PND 4, expression was slightly, but significantly decreased in the HBPA males (B). At PND 10, expression was abrogated in the LBPA males which effectively reversed the expected sex difference in expression compared with vehicle controls (D). Significant differences in expression compared with vehicle are represented by * $P \leq 0.05$ for the females, and # $P \leq 0.05$ for the males. Significant sex differences in expression are represented by & $P \leq 0.05$. The graphs depict mean $\pm$ s.e.m. and the sample size is provided at the bottom (3V, third ventricle; scale bar = 1000 μ m).

levels within the developing brain ostensibly contributes to reported deficiencies in adult sociosexual physiology and behavior in both sexes (NTP 2008, FAO/WHO 2011, Wolstenholme *et al.* 2011, Losa-Ward *et al.* 2012, Rochester 2013). These data also support the hypothesis

posed by us (Adewale *et al.* 2011 #2520) (Patisaul *et al.* 2012 #2605) and others (Wolstenholme *et al.* 2012 #2561; Wolstenholme *et al.* 2011 #2366) that BPA exposure may perturb the organization of OT/AVP signaling pathways.

Figure 7 Autoradiographs depicting *Esr2* signal in the SON and MeA on PND 4 (A) and in the SON (C) and MeA (F) on PND 10. In the SON, EB had no significant effect on *Esr2* expression in either sex, although the levels were no longer distinct enough to be significantly sexually dimorphic. Both doses of BPA decreased *Esr2* signal in PND 4 males, thus diminishing the expected sex difference in expression on PND 4 (B). On PND 10, *Esr2* levels were slightly but significantly decreased in both sexes of the HBPA group (D). In the MeA, EB and both doses of BPA decreased *Esr2* mRNA levels in PND 4 females (E). By PND 10, decreased expression emerged in the HBPA males (G). Effects in the LBPA males were not statistically analyzed due to insufficient sample size. Significant differences in expression compared with vehicle are represented by * $P \leq 0.05$ and ** $P \leq 0.01$ for the females, and # $P \leq 0.05$ and ## $P \leq 0.01$ for the males. Significant sex differences in expression are represented by & $P \leq 0.05$. The graphs depict mean $\pm$ s.e.m. and the sample size is provided at the bottom (3V, third ventricle; scale bar = 1000 μ m for A, and 500 μ m for C and F).

As anticipated, based on prior work by us and others (Kuhnemann *et al.* 1994, Yokosuka *et al.* 1997, Perez *et al.* 2003, Cao & Patisaul 2011, 2013), both *Esr*s were robustly expressed throughout the hypothalamus and surrounding regions on the day of birth, and then diverged in temporal and sexually dimorphic patterns. Importantly, these studies reveal that expression patterns in pre-weanling rats differ from the expression patterns in adults to some degree. In adult rodents, both *Esr* subtypes are present in the BNSTp and MeA (Simerly *et al.* 1990, Kuhnemann *et al.* 1994, Shughrue *et al.* 1997b, Yokosuka *et al.* 1997, Laflamme *et al.* 1998, Osterlund *et al.* 1998, Shughrue & Merchenthaler 2001, Perez *et al.* 2003, Cao & Patisaul 2011, 2013), whereas *Esr2*, but not *Esr1*, is present in the adult PVN, SON, and SCN (Shughrue *et al.* 1997a, 1997b, Osterlund *et al.* 1998, Mitra *et al.* 2003). *Esr1* is the predominant isoform in the arcuate nucleus (ARC) and Pe (Shughrue *et al.* 1997b, Yokosuka *et al.* 1997, Laflamme *et al.* 1998, Osterlund *et al.* 1998, Cao & Patisaul 2011, 2013), and is exclusively expressed in the adult SCN and LHB (Laflamme *et al.* 1998, Vida *et al.* 2008). In this study, sex differences in *Esr2* expression were observed in the Pe and, to a lesser degree, the SON, on PND 19 with expression higher in females. These differences are consistent with morphological and functional sex differences associated with these regions in adults. For example, the volume of SON is larger in adult males than in age-matched female rats (Madeira *et al.* 1993), and the Pe is a component of the region controlling the prenatal gonadotropin surge in females (Mikkelsen & Simonneaux 2009, Poling *et al.* 2013).

The expression patterns were more complex in the BNSTp and PVN. Across postnatal development *Esr2* expression levels generally decreased in the BNSTp but increased in the PVN. Transient sex differences in *Esr2* expression were observed in the BNSTp and PVN (which were lost by PND 19), with males having higher levels than females. Interestingly, a temporary sex difference in *Esr1* expression has also been reported in the rat BNSTp. The levels were observed to be higher in females on PND 6, but this difference was lost on PND 19 and then robustly re-established in adulthood (Kelly *et al.* 2013). In adult BNSTp, both *Esr1* (Kelly *et al.* 2013) and *Esr2* (Zhang *et al.* 2002) expression levels are higher in females. These brief sex differences in expression may contribute to BNSTp-related morphological and functional sex differences found later in life. For example, sex differences in BNSTp volume, cell number, and cell size begin to emerge around PND 7 and result from suppression of cell death in males by estrogen (Murray *et al.* 2009, Hisasue *et al.* 2010, Ahern *et al.* 2013).

Qualitatively, low levels of *Esr2* mRNA were observed in the postnatal SCN of both sexes at all ages and *Esr2* expression was appreciable in the LHB from PND 0–7, then diminished to near the limit of detection by PND 19. Examination of adjacent sections (Cao & Patisaul 2011) revealed minimal signal for *Esr1* in the PND 19

LHB but no *Esr1* signal before that, or at any age in the SCN, suggesting that *Esr2* is the predominant *Esr* isoform expressed in these two regions during postnatal development. The LHB is an important regulatory site of both the midbrain dopamine and dorsal raphe serotonin systems, and integrates information from limbic nuclei (such as the BNSTp and Me) (Reisine *et al.* 1982, Christoph *et al.* 1986, Lecourtier & Kelly 2007, Hikosaka *et al.* 2008) central to reproductive and maternal behavior (Lonstein *et al.* 2000). Previous studies using ovariectomized animals have concluded that only *Esr1* is expressed in the postnatal rat LHB (Yokosuka *et al.* 1997, Laflamme *et al.* 1998, Perez *et al.* 2003, Vida *et al.* 2008), but at least one has reported the presence of both isoforms (Shughrue *et al.* 1997b). In addition to gonadal status, strain differences may also account for the discrepancies between previous studies and the present one.

Neonatal exposure to BPA altered postnatal *Esr2* expression in the BNSTp, SON, MeA, and PVN. When collectively considered with our previous companion study exploring BPA-related *Esr* expression changes in neighboring subnuclei (Cao *et al.* 2012), the data support the hypothesis that one way in which BPA may alter the sex-specific ontogeny of neuroendocrine systems is via perturbation of *Esr* levels in sexually dimorphic brain regions. Differences in local estrogen levels, derived from gonadal androgens in males or synthesized *de novo* (Amateau *et al.* 2004), likely at least partially account for the regional specificity of the effects. The observed gene expression changes could reflect either a change in cellular levels of *Esr2* mRNA within each ROI or a change in the number of cells expressing *Esr2*, the latter of which would suggest that the effect is permanent (McCarthy 2008). Similar studies from our laboratory revealed no significant effects of neonatal BPA (50 µg/kg bw or 50 mg/kg bw) exposure on *Esr1* neuron numbers in the anterior or MBH in adulthood (Patisaul *et al.* 2007, Adewale *et al.* 2011), suggesting that disruption of neonatal *Esr* expression may not manifest as *Esr* neuron loss. Ongoing work in our laboratory is seeking to establish the degree to which BPA-related *Esr* expression level changes persist, if they are accompanied by altered OT/AVP expression changes, and associated with neurobehavioral effects such as altered anxiety (Sullivan *et al.* 2011, Patisaul *et al.* 2012).

Although the BPA doses and route of exposure employed for these studies are not considered human relevant, through work conducted in collaboration with researchers at the National Center for Toxicological Research (NCTR) we have previously shown that oral BPA exposure to the pregnant dam, across a range of environmentally relevant doses (2.5 or 25 µg/kg bw), downregulates *Esr* expression in the neonatal rat hypothalamus and amygdala (Cao *et al.* 2013). In addition, at weaning, the volume of the sexually dimorphic nucleus of the POA (SDN-POA) was significantly larger in the BPA-exposed males, compared with

unexposed conspecifics (He *et al.* 2012). As in the BNSTp, the male SDN is protected from cell death by estrogens derived from gonadal androgens, and is thus larger in males than in females. Enhancement of SDN size by BPA exposure is consistent with an estrogenic mode of action. The health effects of low dose oral exposure remain the subject of considerable interest because human exposure is presumably low but constant and from a variety of sources including food, beverages, the handling of paper receipts, and dust (Vandenberg *et al.* 2007, Biedermann *et al.* 2010, Lakind & Naiman 2010). Our results suggest that one possible outcome may be altered *Esr2* expression in steroid-hormone-sensitive regions of the developing brain, an effect which may have long-term consequences on sociosexual and mood-related behaviors. Further work is needed to better establish if these effects can be induced via exposures that better recapitulate human exposure conditions and doses.

Elucidating the specific mechanisms by which BPA affects neural organization is fundamental for effectively evaluating whether effects observed in rodents can be extrapolated to humans. In humans, the period encompassing the rodent perinatal period is believed to occur in mid to late gestation (Selevan *et al.* 2000, Simerly 2002, Aksglaede *et al.* 2006, Abbott *et al.* 2008); thus, the rat perinatal 'critical window' is likely to be entirely prenatal in humans. The possible health consequences of BPA exposure remain controversial (Goodman *et al.* 2009, Vandenberg *et al.* 2009, Beronius *et al.* 2010), but there is growing concern that early-life exposure may alter neural development and ultimately contribute to neurobehavioral disorders in humans (vom Saal *et al.* 2007, Chapin *et al.* 2008, NTP 2008, Palanza *et al.* 2008, Patisaul & Polston 2008, Report of Joint FAO/WHO Expert Meeting 2011, Wolstenholme *et al.* 2011, Rosenfeld 2012). Weight of evidence assessments have been conducted by numerous groups, but conclusions regarding the degree of concern that consumers should have about BPA have been inconsistent (vom Saal *et al.* 2007, NTP 2008, Hengstler *et al.* 2011). This study is novel because it provides new insight as to how BPA may be influencing brain organization. Although there are critical species differences specific to how estrogen organizes the developing brain (Resko & Roselli 1997, McCarthy 2008), the distribution of sex-specific *Esr* expression is well conserved across species (including humans) (MacLusky *et al.* 1979, Brandenberger *et al.* 1997, Resko & Roselli 1997, Kato *et al.* 1998, Wallen 2005, Gonzalez *et al.* 2007, Walker *et al.* 2009, Cao & Patisaul 2013). *Esr2*, particularly in the PVN, is important for modulating affective and mood-related behaviors, including anxiety, aggression, and social interactions (Lund *et al.* 2005, Patisaul & Bateman 2008, Handa *et al.* 2012). Moreover, the ROIs examined in this study are critical components of AVP/OT signaling pathways and related systems crucial for mediating aspects of sociosexual behavior including affiliation and sociality (Neumann &

van den Burg 2011). Thus a potential outcome of *Esr2* disruption in the ROIs examined in this study is altered anxiety and social behaviors in later life. This hypothesis is consistent with previous work in our laboratory demonstrating that oral exposure to BPA across perinatal development, at levels considered to be human-relevant, resulted in abrogated *Esr2* expression in the adolescent amygdala, as well as elevated juvenile anxiety (Patisaul *et al.* 2012). Numerous other studies have also reported behavioral impacts of early-life BPA exposure, including elevated anxiety, in a wide range of species (Jasarevic *et al.* 2011, Wolstenholme *et al.* 2011, Rosenfeld 2012, Jasarevic *et al.* 2013, Kundakovic *et al.* 2013). In young children, developmental BPA exposure has been associated with hyperactivity and elevated anxiety (Braun *et al.* 2011, Harley *et al.* 2013) but whether BPA exposure may contribute to neurobehavioral and mood disorders remains unknown. These data contribute important information regarding the mechanisms by which BPA-related behavioral changes may emerge and implicate disruption of the AVP/OT system. This putative link should be addressed in future experimental work, and further assessments of BPA-related impacts on neurobehavior in humans should be conducted with this potential association in mind.

Discussion from meeting

Emilie Rissman (Charlottesville, USA): In your gavage studies can you examine direct 'stress' target genes such as *Crh* or *Gr* in the offspring amygdala?

Heather Patisaul (Raleigh, USA): To do that would require permission from our collaborators at NCTR (National Center for Toxicology Research). Also, to more comprehensively confirm that the gene expression changes are caused by gavage, and not the vehicle, the experiment should be replicated and include animals treated with vehicle without gavage. That control group is missing from the present experiments.

Alana Sullivan (Raleigh, USA): The animals in your first study had a soy-free diet, and you used a soy diet in the second experiment. Diet is an important factor as a possible cause of endocrine disruption.

Heather Patisaul: Diet is part of the equation that we are working on and needs further exploration. There are important differences between soy and casein diets. It is possible that the phytoestrogens in soy interfere with the BPA action. Diet is an environmental factor to consider. Some ESRs (ESR2) are sensitive to soy.

Jane Muncke (Food Packaging Forum, Zurich, Switzerland): What equipment did you use for gavage? If it was plastic, did you test for leaching of endocrine-disrupting chemicals (EDCs) into the vehicle oil?

Heather Patisaul: For our studies done in collaboration with NCTR, the gavage equipment is all metal with no plastic and it is extensively tested along with the BPA mixture, the glass bottles, the vehicle, and the inside of the gavage machine. The machine weighs the animals

and automatically dispenses the correct amount of vehicle. There is no contamination from this equipment as far as I know.

Jane Muncke: How is the feed packaged? This might be a source of EDC migration.

Heather Patisaul: For those studies, feeding is done at NCTR and I am not sure how it is packaged on bulk arrival. For the present studies, we received the feed in cardboard boxes and I do not know if they are lined with anything that contains BPA. We did not test the feed in previous experiments, but in current, ongoing studies with NCTR the feed has been tested and not found to be contaminated.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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