SEX AND TIME DEPENDENT EFFECTS OF DEVELOPMENTAL PBDES ON HYPOTHALAMO-PITUITARY-THYROID AXIS ARE PARTIALLY RESTORED BY MATERNAL LEVOTHYROXINE SUPPLEMENTATION IN C57BL/6 MICE

Kozlova EV[1], De Angelis M[2], Denys ME[1], Schramm K-W[2,3] and MC Curras-Collazo[1]

[1]University of California Riverside, Dept. of Molecular, Cell and Systems Biology, Riverside, California, USA, 92507, ekozl001@ucr.edu; [2]Helmholtz Zentrum Munchen, German National Research Centre for Environmental Health, Neuherberg, Munich, Germany; [3]TUM, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Department für Biowissenschaftliche Grundlagen, Freising, Germany

Introduction

Polybrominated diphenyl ethers (PBDEs) are persistent organic pollutants (POPs) with known endocrine disrupting and neurotoxic effects[1],[2]. PBDEs have been widely used as flame retardants since the 1970s, though a global ban at the Stockholm Convention and voluntary phase-out in the US have been effective in gradually declining environmental and human levels. Nevertheless, PBDEs are estimated to continue to contaminate biota through 2050 due to e-waste and inadvertent recycling[3],[4]. PBDEs have been recently detected in indoor dust samples at levels that elicit hormonal activity towards human nuclear hormone receptors,[5] as well as in various tissue samples world-wide[6],[7]. PBDE exposures during periods of biological plasticity such as fetal and early postnatal development are of particular concern. PBDEs detected in maternal serum and breastmilk are associated with impairments in executive function, psychomotor development, attention and social competence in children[8]. PBDEs can cross the blood-brain barrier and accumulate in the central nervous system[9], however, the potential mechanisms of how PBDEs may impair brain development are understudied.

Thyroid hormones (THs) are essential epigenetic factors for regulating the precise spatiotemporal development of the brain. Animal studies of TH deficiency produced by administration of the anti-thyroid drugs such as propylthiouracil (PTU) have shed light on the developmental actions of THs[10]. Functional neuronal circuits are assembled through the TH-mediated progressive events[11] of neurogenesis, cell migration, synaptogenesis, formation of cortical layers, neuronal and glial differentiation and myelination[12]. Experimental hypothyroidism during specific fetal stages leads to irreversible deficiencies such as altered in cell migration, resulting in less defined cortical layering, altered corpus callosum connections, decreased cells in the dentate gyrus of the hippocampus and delayed cerebellar development[13]. Behaviorally, these changes result in reduced spatial learning[14], auditory[15] and motor deficits[16] and impaired maternal behavior[17]. These animal studies recapitulate human studies and reinforce the concept of a critical time window when TH is required for normal brain development, during which insufficient TH can induce long-term intellectual and behavioral impairments[18].

Studies in humans and animals have provided evidence that the hypothalamic-pituitary-thyroid (HPT) axis is particularly vulnerable to disruption by PBDEs,[9],[19] which bear structural homology to THs. The numerous steps required for TH synthesis and metabolism allows PBDEs to potentially dysregulate the HPT axis at multiple functional levels, producing complex physiological effects. For instance, PBDEs can interfere with deiodinase (DIO) enzyme activity/expression, enhance the metabolism and elimination of THs, alter the expression and activity of plasma and membrane bound transporters, thereby affecting tissue uptake, and alter genomic signaling[19],[20]. Human studies examining the association between PBDE body burdens and plasma THs have found positive[21],[22] and negative correlations,[23],[24] while most animal studies have shown that PBDEs reduce serum Thyroxine (T4) concentrations[9]. In contrast to the monotonic response of PTU to decrease serum thyroxine (T4), leading to subsequent T4 reductions in target tissues, followed by pituitary feedback to increase plasma thyroid-stimulating hormone (TSH), PBDEs can perturb the HPT axis in less predictable ways[25]. T4, which is made by the thyroid gland, can enter the brain and is converted to an active form, triiodothyronine (T3). Thus, plasma TH levels are not necessarily indicative of PBDEs actions in target tissues such as the brain. Whether plasma TH levels are a proxy for brain thyroid status, has not been studied in a rodent PBDE model.

In this study, we investigated the effects of a commercial PBDE mixture, DE-71, on brain THs following maternal exposure from early gestation through lactation, alone and combination with T4 supplementation. A group of dams receiving PTU was included as a positive control. We tested the hypothesis that developmental PBDE exposure dysregulates brain levels of distinct TH species and examined if PBDE action on plasma T4 would be indicative of its downstream effects in brain tissue. Male and female F1 offspring were examined to determine sex-specific effects at two developmental time points. The effects of T4 supplementation on plasma T4 and body weight were also examined. Since thyroid hormones can regulate the social neuropeptides oxytocin (OXT) and vasopressin (AVP)[26], which are altered by organohalogens,[27],[28] we measured plasma OXT levels and maternal behavior in dams. Our findings suggest complex interactions between PBDEs and plasma and brain levels of THs which may help elucidate the relationship between serum and brain TH levels in developing

offspring. These findings may have implications for neurodevelopmental disorders and inform legislative regulation of environmental chemicals with thyroid disrupting action.

Materials and methods

Animal Model: Animals were housed in a controlled environment: temperature (21.1–22.8°C), light (12:12 light–dark; lights on at 0800 h), and humidity (20-70%). Mice had access to food and water *ad libitum*. Care and treatment of animals was performed in compliance with guidelines from NIH and approved by the University of California Riverside Institutional Animal Care and Use Committee. The dosing paradigm, which included *in utero* and lactational exposure to the penta PBDE mixture, DE-71, has been previously reported[29]. In brief, C57Bl6/N mouse dams (F0) were exposed to DE-71 via oral administration of 0 (VEH/CON), 0.1 (L-DE71) or 0.4 (H-DE71) mg/kg bw/d from 3 wk prior to gestation (GD) through end of lactation on postnatal day (PND) 21. Another group of dams were treated with T4 at 8 μg/100 g bw (GD 12-PND 21) in water, as described[30]. A subset of VEH/CON dams were administered PTU (50 mg/L) in drinking water GD 14-PND 21[17],[31]. ***Brain TH Determination:*** Mice were sacrificed under isoflurane anesthesia followed by cervical dislocation. Whole brains were rapidly dissected and flash-frozen. Samples were homogenized at -200°C to powder from which ~100 mg was extracted for determination of THs. The standard solutions and clean-up procedure was performed as previously described[32]. Analysis of seven THs: T4, T3, reverse 3,3',5'-triiodothyronine (rT3), 3,3´-diiodo-L-thyronine (T2), 3,5-diiodo-L-thyronine (rT2), 3-iodo-L-thyronine (T1) and 3-iodothyronamine (T1AM) was performed with the Agilent 1290 Infinity II LC system interfaced with an Agilent 6470 triple quadrupole tandem mass spectrometer, as previously described[33]. Data acquisition, linearity of the standard curve and quantification of the samples were performed using Agilent MassHunter Workstation. ***Plasma Thyroid and Oxytocin EIA:*** Plasma total T4 and OXT were quantified by commercial colorimetric kits (K050-H1, K048-H1, Arbor Assays) following manufacturer instructions. Prior to assaying, samples were processed with the supplied extraction solution. Sample concentrations were measured spectrophotometrically at 450 nm using a standard curve of known concentrations. ***Pup Retrieval Test:*** PND 2-7 pups were isolated from their mothers for 20 min with a heating pad at 35°C. Three pups were placed in the corners of the dam's home cage. Maternal behaviors including latency to investigate the first pup and duration taken to return all 3 pups into the nest was scored *a posteriori* from video recordings[34]. ***Statistical Analyses:*** Statistical analysis was performed using GraphPad Prism (version 8.4.3). Within groups comparison was performed using Student's t-test or one or two-way ANOVA if more than two groups were compared.

Results and discussion

DE-71 exposure leads to sex- and age-specific alterations in brain and plasma THs

Plasma total T4 (TT4) was significantly reduced in L-DE71 males at PND 15 relative to VEH/CON but this effect was normalized by PND 30 (**Fig. 1A-B**). Results reported by Bansal and others (2014) show no effects of perinatal DE-71 on plasma T4 in male rats, supporting a time-dependence of DE-71 effects[24]. T4 supplementation elevated plasma TT4 in males only in the VEH/CON group at PND 30 vs corresponding controls (p<.001). In contrast, no DE-71 exposure group in males responded to supplementation by increasing plasma TT4. In females, there was no exposure effect of DE-71 on plasma TT4. However, T4 supplementation produced a significant rise in plasma TT4 compared to unsupplemented VEH-CON (p<.001), L-DE71 and H-DE71 controls (p<.05). Taken together, supplementation was effective in raising plasma TT4 in a sex- and time-dependent manner, i.e, only in females at PND 30. For brain TH levels DE-71-exposed offspring showed several alterations as compared to VEH/CON. We observed sex- and age-dependent differences in brain levels of T3, T4, rT3 and T2 (**Fig. 1C-F**). We were not able to detect the lower iodinated analogues, rT2 and or T1 nor the novel decarboxylated metabolite T1AM[35] using this methodology. In PND 30 males, there was a non-monotonic effect on T4; L-DE71 produced a reduction and H-DE71 produced a significant elevation in T4 levels relative to VEH/CON (p=.05). H-DE71 also elevated rT3 and T2. No changes were observed at PND 15. L-DE71 exposed females showed changes in several brain THs measured at PND 15, namely, reduced T4, rT3 and T2 in L-DE71 (p<.05-.001) and elevated T3 in H-DE71 group. These differences were normalized by PND 30 except for elevated T2 in H-DE71. In both males and females, brain T4 followed by T3 were more abundant than rT3 and T2 as expected, since these are deactivation products of T4 and T3 by DIO3, respectively. Moreover, DE-71-induced changes in female F1 brain T4 levels did not parallel changes in plasma TT4, while in males there was a predictable but delayed response following a drop in the plasma. This suggests that plasma TT4 status is not always indicative of brain thyroid status and that there are differential time windows of effects in males and females.

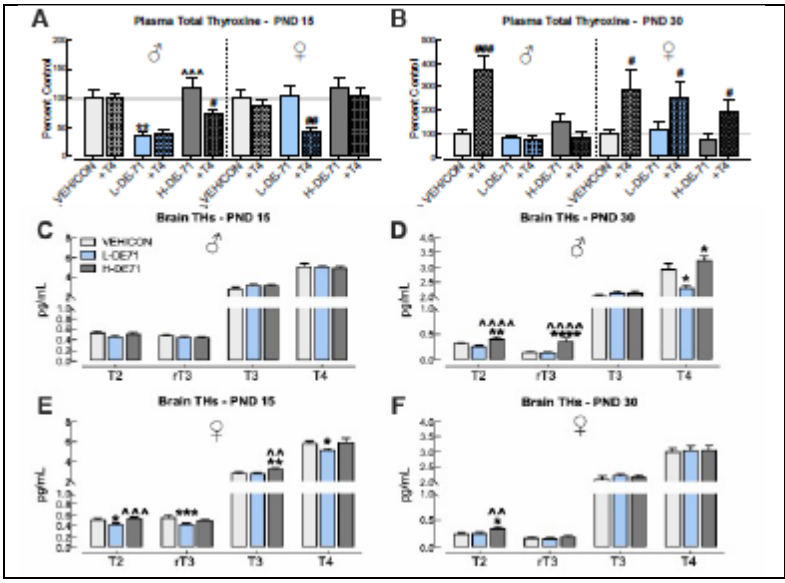

Figure 1. Effects of DE-71 exposure on offspring brain and plasma TH levels. TH hormones were quantified in plasma (**A-B**) whole brain tissue (**C-D**) of **offspring** of dams exposed to L-DE-71 or H-DE-71 and compared to VEH/CON. **p*<.05, ***p*<.01, ****p*<.001, *****p*<.0001 compared to VEH/CON; #*p*<.05, ###*p*<.001 compared to corresponding control; ^^*p*<.01, ^^^*p*<.001, ^^^^*p*<.0001 compared to L-DE-71. *n* subjects/group= 4-10 (a-d), 3-6 (e), 5-9 (f).

Reduced body weight gain produced by DE-71 is ameliorated by maternal T4 supplementation

Reduced body weight following perinatal exposure to high doses of DE-71 has been reported[9]. In male and female mice, pup body weights increased steadily with age in all exposure groups as expected (**Fig. 2A-D**). H-DE71 males and females showed reduced body weight gain relative to VEH/CON (**Fig. 2A,C**). Weight reduction was initiated earlier in females (PND 12), while in males, it was first observed at PND 20. These critical times were delayed in L-DE71 groups: females affected from PND 16 and males at PND 23. These results indicate that females are more sensitive to the growth-reducing actions of PBDEs and correspond to the differential timing of PBDE reducing actions on THs in the brain (**Fig. 1**). T4 supplementation normalized body weight in L-DE71 but not H-DE71 offspring of both sexes examined at PND 8-30 (**Fig. 2B-D**). Body weight in supplemented males showed increased (L-DE71) and decreased body weight (H-DE71) as compared to VEH/CON. T4 supplementation increased body weight gain in L-DE71 females. Coincidently, T4 supplementation increases plasma T4 in female PND 30 which may explain normalization of body weight seen PND 23-30 due to somatic trophic effects of thyroid hormone[36]

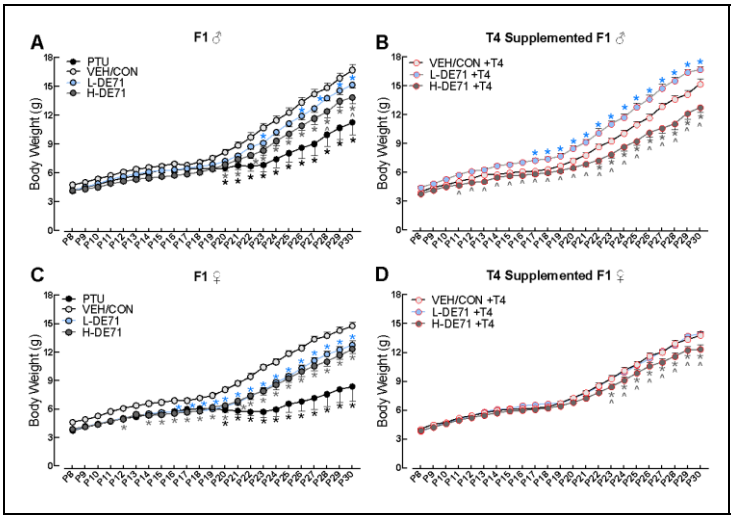

Figure 2. Levothyroxine supplementation given to dams rescues postnatal body weight changes in males and female offspring exposed to 0.1 but not 0.4 mg/kg DE-71. Body weights of offspring were recorded from PND 8-30 and compared to VEH/CON. **p*<.05-.0001 compared to VEH/CON; ^*p*<.05-.0001 compared to L-DE71. *n*= 12-18 except PTU, which had 5-6 subjects/group.

Although plasma T4 levels also increased in T4 supplemented H-DE71 females, body weight was not restored, suggesting differential thyroid responsiveness in L- and H-DE71 exposed female offspring. PTU treatment given to dams caused a dramatic decrease in body weight gain in female and male offspring relative to VEH/CON from PND 20-30. Taken together, these results suggest that the response to developmental DE-71 exposure on this metric is monotonic, that females are more sensitive during the developmental window examined and phenotypic rescue is possible by maternal T4 supplementation in the L-DE71 groups of both sexes.

Perinatal DE-71 exposure does not reduce maternal behavior nor plasma oxytocin

To examine effects of PBDE, T4 and PTU treatments on maternal parameters, we examined maternal behavior and plasma oxytocin that is critically important for offspring postnatal development. There was no effect of PBDE treatment on plasma OXT levels in dams; however, these levels increased in T4 supplemented dams (**Fig. 3A**). A regulatory effect of T4 on plasma OXT has been previously shown in female mice and male rats likely due to enhancement of OXT mRNA transcripts[26],[37]. There was no effect of developmental exposure to DE-71 on the pup retrieval test. In contrast, PTU dams took significantly longer to identify the first pup (**Fig. 3C**). Supplemented L-DE-71 dams showed increased latency to first pup (p<.05). These results suggest that the PBDE effects on offspring TH or body weight gain during postnatal development are not likely due to lack of maternal care.

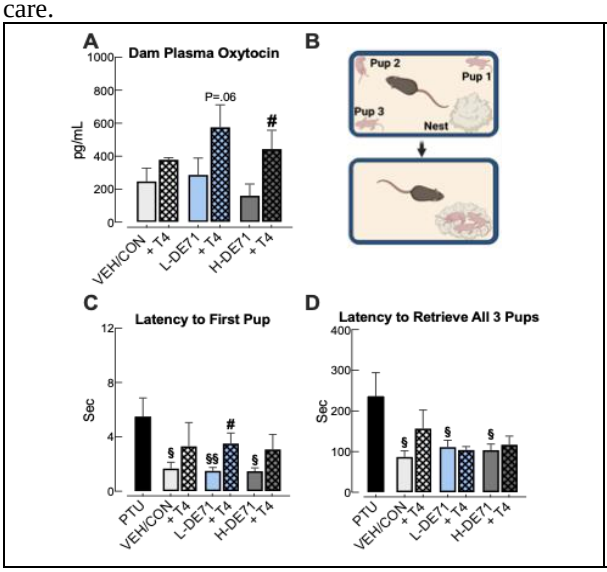

Figure 3. DE-71 Effects on Maternal Behavior and Plasma Oxytocin. (**A**) Plasma oxytocin levels in dams taken at time of weaning. (**B**) Schematic of the pup retrieval test. (**C**) The latency to identify the first pup during the pup retrieval test. PTU treated dams spent significantly longer time to identify the first pup. (**D**) The latency to retrieve all three pups during the 10 min test time. #P<.05, ##P<.01 compared to corresponding exposure control; §p<.05 compared to PTU; n=subjects/group: 2-6 (a), 4-10 (c-d).

We report, for the first time, the effect of maternal transfer of DE-71 on brain THs in offspring. Brain TH species are likely to influence brain growth and maturation during critical perinatal developmental stages[12]. We found age- and sex-dependent DE-71-induced hypothyroidism, i.e., reduced plasma T4 levels were seen only in males at PND 15. Interestingly, in 3 year-old boys but not girls, prenatal BDE-47 and BDE-99 are inversely related to free T4[38]. Further, peripheral and central thyroid status were affected differently by DE71 in both males and females. Female brains showed altered THs at PND 15, while males showed delayed changes at PND 30. However, males and females showed dissimilar patterns for altered TH species. For example, while T2, rT3, T4 were elevated in H-DE71 males and T4 was apparently reduced in L-DE71 males, females showed lowered T4, T2, rT3 in L-DE71 and elevated T3 in H-DE71; only a drop in brain T4 in L-DE71 and a rise in T2 in H-DE71 was common to both. From these findings, we can conclude that brain levels of T4 don't reflect serum T4 levels. The differential effects of L-DE-71 on brain but not plasma THs was especially notable in females where lowered brain T4 and rT3 were not matched by lower plasma T4 indicating possible inhibitory actions of PBDEs on brain thyroid transporters and/or local DIOs[39],[30]. Results obtained using PTU treatment indicated a different pathophysiology compared to that of DE-71 exposure. However, the cause(s) of altered brain THs in both sexes is unclear. It will be important to relate the findings in offspring to possible hypothyroid effect of PBDEs in dams during pregnancy since overt hypothyroidism in pregnant mothers can lower IQ and impair brain development in their children[40]. Therefore, our novel findings on the effects of maternal T4 supplementation on the thyroid axis deficits of DE-71 exposed offspring will yield impactful findings that can be used to understand more fully the actions of PBDEs and other thyroid-disrupting chemicals on neurodevelopment and NDDs.

Acknowledgements

We are grateful to Dr. Sladek (UC Riverside) lab for the gift of mice. We acknowledge support from UCR GRMP (E.V.K.) and COR grant (M.C.C.). Images were created with Biorender.com.

References

[1] L. G. Costa, R. de Laat, S. Tagliaferri, C. Pellacani, *Toxicol. Lett.* **2014**, *230*, 282.

[2] E. V. Kozlova, M. C. Valdez, M. E. Denys, A. E. Bishay, J. M. Krum, K. M. Rabbani, V. Carrillo, G. M. Gonzalez, G. Lampel, J. D. Tran, B. M. Vazquez, L. M. Anchondo, S. A. Uddin, N. M. Huffman, E. Monarrez, D. S. Olomi, B. D. Chinthirla, R. E. Hartman, P. R. S. Kodavanti, G. Chompre, A. L. Phillips, H. M. Stapleton, B. Henkelmann, K.-W. Schramm, M. C. Curras-Collazo, *Arch. Toxicol.* **2022**, DOI 10.1007/s00204-021-03163-4.

[3] C. M. Ohajinwa, P. M. Van Bodegom, Q. Xie, J. Chen, M. G. Vijver, O. O. Osibanjo, W. J. G. M. Peijnenburg, *Int. J. Environ. Res. Public Health* **2019**, *16*, DOI 10.3390/ijerph16030360.

[4] G. Abbasi, L. Li, K. Breivik, *Environ. Sci. Technol.* **2019**, *53*, 6330.

[5] A. S. Young, T. Zoeller, R. Hauser, T. James-Todd, B. A. Coull, P. A. Behnisch, A. Brouwer, H. Zhu, K. Kannan, J. G. Allen, *Environ. Health Perspect.* **2021**, *129*, 47010.
[6] P. Terry, C. V. Towers, L.-Y. Liu, A. A. Peverly, J. Chen, A. Salamova, *Int. J. Environ. Health Res.* **2017**, *27*, 205.
[7] S. Hurley, D. Goldberg, D. O. Nelson, W. Guo, Y. Wang, H.-G. Baek, J.-S. Park, M. Petreas, L. Bernstein, H. Anton-Culver, P. Reynolds, *Environ. Sci. Technol.* **2017**, *51*, 4697.
[8] E. A. Gibson, E. L. Siegel, F. Eniola, J. B. Herbstman, P. Factor-Litvak, *Int. J. Environ. Res. Public Health* **2018**, *15*, DOI 10.3390/ijerph15081636.
[9] P. R. S. Kodavanti, C. G. Coburn, V. C. Moser, R. C. MacPhail, S. E. Fenton, T. E. Stoker, J. L. Rayner, K. Kannan, L. S. Birnbaum, *Toxicol. Sci.* **2010**, *116*, 297.
[10] R. T. Zoeller, K. M. Crofton, *Crit. Rev. Toxicol.* **2005**, *35*, 771.
[11] M. M. Riccomagno, A. L. Kolodkin, *Annu. Rev. Cell Dev. Biol.* **2015**, *31*, 779.
[12] G. W. Anderson, C. M. Schoonover, S. A. Jones, *Thyroid* **2003**, *13*, 1039.
[13] P. Berbel, E. Ausó, J. V. García-Velasco, M. L. Molina, M. Camacho, *Neuroscience* **2001**, *107*, 383.
[14] M. C. Opazo, A. Gianini, F. Pancetti, G. Azkcona, L. Alarcón, R. Lizana, V. Noches, P. A. Gonzalez, M. P. Marassi, S. Mora, D. Rosenthal, E. Eugenin, D. Naranjo, S. M. Bueno, A. M. Kalergis, C. A. Riedel, *Endocrinology* **2008**, *149*, 5097.
[15] E. S. Goldey, L. S. Kehn, G. L. Rehnberg, K. M. Crofton, *Toxicol. Appl. Pharmacol.* **1995**, *135*, 67.
[16] M. A. Khairinisa, Y. Takatsuru, I. Amano, M. Kokubo, A. Haijima, W. Miyazaki, N. Koibuchi, *Int. J. Clin. Pharmacol. Res.* **2019**, *4*, DOI 10.15416/pcpr.v4i3.25264.
[17] M. A. Khairinisa, Y. Takatsuru, I. Amano, M. Kokubo, A. Haijima, W. Miyazaki, N. Koibuchi, *Front. Endocrinol.* **2018**, *9*, 228.
[18] R. T. Zoeller, J. Rovet, *J. Neuroendocrinol.* **2004**, *16*, 809.
[19] P. R. S. Kodavanti, M. C. Curras-Collazo, *Front. Neuroendocrinol.* **2010**, *31*, 479.
[20] P. D. Noyes, H. M. Stapleton, *Endocrine Disruptors* **2014**, *2*, e29430.
[21] M. E. Turyk, V. W. Persky, P. Imm, L. Knobeloch, R. Chatterton, H. A. Anderson, *Environ. Health Perspect.* **2008**, *116*, 1635.
[22] S. C. Byrne, P. Miller, S. Seguinot-Medina, V. Waghiyi, C. L. Buck, F. A. von Hippel, D. O. Carpenter, *Sci. Rep.* **2018**, *8*, 2198.
[23] Y. Oulhote, J. Chevrier, M. F. Bouchard, *J. Clin. Endocrinol. Metab.* **2016**, *101*, 590.
[24] J. B. Herbstman, A. Sjödin, B. J. Apelberg, F. R. Witter, R. U. Halden, D. G. Patterson, S. R. Panny, L. L. Needham, L. R. Goldman, *Environ. Health Perspect.* **2008**, *116*, 1376.
[25] R. Bansal, D. Tighe, A. Danai, D. F. K. Rawn, D. W. Gaertner, D. L. Arnold, M. E. Gilbert, R. T. Zoeller, *Endocrinology* **2014**, *155*, 4104.
[26] R. A. Adan, J. J. Cox, J. P. van Kats, J. P. Burbach, *J. Biol. Chem.* **1992**, *267*, 3771.
[27] S. Mucio-Ramírez, E. Sánchez-Islas, E. Sánchez-Jaramillo, M. Currás-Collazo, V. R. Juárez-González, M. Y. Álvarez-González, L. E. Orser, B. Hou, F. Pellicer, P. R. S. Kodavanti, M. León-Olea, *Toxicology and Applied Pharmacology* **2017**, *329*, 173.
[28] M. P. Reilly, M. N. Kunkel, L. M. Thompson, A. Zentay, C. D. Weeks, D. Crews, L. K. Cormack, A. C. Gore, *J. Exp. Zool. A Ecol. Integr. Physiol.* **2021**, DOI 10.1002/jez.2475.
[29] E. V. Kozlova, B. D. Chinthirla, P. A. Pérez, N. V. DiPatrizio, D. A. Argueta, A. L. Phillips, H. M. Stapleton, G. M. González, J. M. Krum, V. Carrillo, Others, *Sci. Rep.* **2020**, *10*, 1.
[30] S. Bárez-López, M. J. Obregon, J. Bernal, A. Guadaño-Ferraz, *Cereb. Cortex* **2018**, *28*, 1783.
[31] I. Amano, Y. Takatsuru, M. A. Khairinisa, M. Kokubo, A. Haijima, N. Koibuchi, *Endocrinology* **2018**, *159*, 1910.
[32] Z.-M. Li, D. Hernandez-Moreno, K. M. Main, N. E. Skakkebæk, H. Kiviranta, J. Toppari, U. Feldt-Rasmussen, H. Shen, K.-W. Schramm, M. De Angelis, *Endocrinology* **2018**, *159*, 3473.
[33] Z.-M. Li, M. Miller, S. Gachkar, J. Mittag, S. C. Schriever, P. T. Pfluger, K.-W. Schramm, M. De Angelis, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2021**, *1165*, 122553.
[34] W.-L. Wu, C.-H. Wang, E. Y.-K. Huang, C.-C. Chen, *PLoS One* **2009**, *4*, e6508.
[35] T. S. Scanlan, *Endocrinology* **2009**, *150*, 1108.
[36] A. J. Forhead, A. L. Fowden, *J. Endocrinol.* **2014**, *221*, R87.
[37] J. P. Stohn, M. E. Martinez, M. Zafer, D. López-Espíndola, L. M. Keyes, A. Hernandez, *Genes Brain Behav.* **2018**, *17*, 23.
[38] A. M. Vuong, J. M. Braun, G. M. Webster, R. Thomas Zoeller, A. N. Hoofnagle, A. Sjödin, K. Yolton, B. P. Lanphear, A. Chen, *Environ. Int.* **2018**, *117*, 339.
[39] A. C. Schroeder, M. L. Privalsky, *Front. Endocrinol.* **2014**, *5*, 40.
[40] D. Levie, T. I. M. Korevaar, S. C. Bath, A. Dalmau-Bueno, M. Murcia, M. Espada, M. Dineva, J. M. Ibarluzea, J. Sunyer, H. Tiemeier, M. Rebagliato, M. P. Rayman, R. P. Peeters, M. Guxens, *J. Clin. Endocrinol. Metab.* **2018**, *103*, 2967.