NEUROTOXIC EFFECTS OF DEVELOPMENTAL EXPOSURE TO DE-71 ON FOREBRAIN SOCIAL PEPTIDES, SOCIAL BEHAVIOR AND OLFACTION IN C57BL/6 MICE

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Abstract: Polybrominated diphenyl ethers (PBDEs) are indoor flame retardant pollutants that produce adverse neurobehavioral effects likely due to endocrine disrupting and neurotoxicological actions. In humans, PBDEs are associated with impairments in executive function, psychomotor and mental development, hyperactivity, poorer attention and in some cases social competence. Results from studies on experimental animals that were exposed to PBDEs during development, indicate that PBDE exposure during critical windows of susceptibility can alter brain processes and behavior in adulthood. Our lab has found that perinatal exposure to DE-71, an industrial mixture of PBDEs, produces a behavior phenotype characterized by reduced social recognition ability and altered anxiety in adult male offspring. To examine if PBDEs produce sexually dimorphic effects, this study used females to evaluate affective behaviors and gene expression of prosocial peptides, oxytocin and vasopressin and their receptors, critical components of the social recognition pathway. Furthermore, effects of adult exposure to DE-71 were also examined using mothers of abnormally behaving offspring. C57BL/6 dams were dosed with DE-71 for 10 weeks (preconception: 4 weeks; gestation: 3 weeks; lactation: 3 weeks). Dosing consisted of low dose (LD; 0.1 mg/kg/d), high dose (HD; 0.4 mg/kg/d), or corn oil vehicle control (OD) administered via ingestion of infused corn flakes. Maternal food and water intake and sex ratio of offspring were not affected, but LD litter size was reduced. PBDE congener accumulation in the brain of female offspring, as detected by GC/ECNI-MS, was limited to BDE 100 and 153. Exposed dams and their female progeny were subjected to behavioral testing for social memory (social recognition task-SRT), olfaction (olfactory habituation/dishabituation-OHP and olfactory preference test-OPT), anxiety (elevated plus maze-EPM and Suok) and depressive-like behavior (forced swim test-FST). When compared to OD controls, LD and HD female offspring exhibited deficits in SRT ability, showing no preference to investigate a novel versus familiar mouse. DE-71 at LD perturbs the ability to discriminate between two social odors as measured by an olfactory habituation/dishabituation test (OHT) and preference for peanut butter over butyric acid on the olfactory preference test (OPT). On EPM, HD offspring showed an apparent increase in time spent in closed arm as well as an increased latency to leave center on Suok as compared to OD indicating greater anxiety. In contrast, there was no effect of treatment on depressive-like behavior as measured by FST. Interestingly, adult exposure in LD and HD dams did not produce altered behavior. In the brains of mice used for behavior testing, gene expression of vasopressin (*Avp, Avp1ar*) and oxytocin signaling (*Oxt, Oxtr*), which serve as markers for social recognition behavior and anxiety, were examined. In HD female offspring we found significantly reduced *Avp* in paraventricular (PVN) and supraoptic nuclei of the hypothalamus (SON) and *Avp1ar* expression in the SON. DE-71-induced changes in the OXTergic system were also detected such as reduced *Oxt* in LD and HD female offspring in SON and increased *Oxtr* in PVN of LD females. Our findings indicate that developmental exposure to environmentally relevant PBDE doses and congeners perturbs vasopressin and oxytocin neurochemical circuits that may underlie altered social recognition and anxiety behaviors in adulthood. Moreover, affective behaviors are susceptible to DE-71 only if exposure occurs during perinatal development but not adulthood.

Introduction: Polybrominated Diphenyl Ethers (PBDEs) are brominated flame retardants that disrupt endocrine, neuroendocrine and neural function.[1] PBDEs have been linked to adverse neurobehavioral effects such as lower psychomotor and mental development, poorer attention, reduced social competence, altered externalizing behaviors and increased psychological stress and anger.[2,3,4,5,6,7,8,9] Children are at greater risk of adverse health effects due to additional routes of exposure, most notably via maternal transfer of contaminated breast milk.[10] Additionally, an industrial mixture of PBDE congeners, DE-71, added to upholstery and furniture, can affect toddlers and young children via contaminated dust that is ingested and/or dermally absorbed and via contaminated food.[11,12] Results from studies on experimental animals that are developmentally exposed to PBDEs support an association between PBDEs and neurobehavioral deficits related to learning and locomotion but little is known about their effects on social behavior.[13,14] We and others have found that neuroendocrine factors regulating social behaviors such as pituitary adenylate cyclase activating polypeptide, and vasopressin (and signaling partners such as nitric oxide and calcium buffering), are disrupted by PBDEs and/or their structural and functional analogues, polychlorinated biphenyls.[15,16,17] For example, rat pups exposed *in utero* and during lactation with DE-71 given orally to their mothers via popcorn can nearly abolish vasopressin immunoreactivity in the supraoptic and paraventricular nuclei of the hypothalamus.[18] Therefore, abnormal social behavior produced by perinatal PBDEs

may be due to reduced content of the social peptides, vasopressin (and/or oxytocin) or their receptors. Interestingly, the promoter regions for both neuropeptides vasopressin and oxytocin are susceptible to epigenetic modification via methylation[19] and PBDEs can induce epigenetic changes, global DNA hypomethylation, in the mouse brain after exposure *in utero.*[20] Therefore, we hypothesize that developmental PBDE exposure can alter the expression of neuropeptides that may lead to abnormal social behavior phenotypes relevant to neurobehavioral disorders. Given the accelerated rise in the incidence of the neurodevelopmental disorder autism spectrum disorder (ASD)[21], it has been speculated that persistent organic pollutants may act as environmental risk factors for its etiology.[22,23] However, additional studies are needed to determine whether PBDEs are associated with ASD[8]. In this study, we demonstrate a correlation between perinatal exposure to low concentrations of DE-71 and social behavior phenotype, olfactory function and gene expression profiles of "social" peptides, vasopressin and oxytocin in discrete brain regions that control social behavior. We use an industrial mixture of PBDEs, DE-71, to mimic chronic low-level exposure to chemical mixtures encountered by humans.[8]

Materials and Methods

Developmental exposure to DE-71: C57Bl/6 mice dams were orally dosed with control corn oil vehicle (Oil), low dose (LD) (0.1 mg/kg/d), or high dose (HD) (0.4 mg/kg/d). Dams were dosed for 10 weeks (4 weeks of preconception, 3 weeks of gestation, and 3 weeks of lactation). One exception was made on the day of birthing when dams were undisturbed. Female offspring were evaluated in adulthood at PND 40-55 for Suok beam test, PND 60-90 for Social Recognition and Social Novelty Tests, PND 80-110 for Olfactory Habituation/Dishabituation and Olfactory Preference Tests and PND 110-120 for Elevated Plus Maze and Forced Swim Tests and soon after sacrificed under isoflurane anesthesia for brain harvest. Dams underwent the battery of behavioral testing at least one week post weaning of pups with 3-5 day rest between tests.

DE-71 Congener analysis via GC/ECNI-MS: Brain tissue samples were obtained from adult female offspring at sacrifice and frozen at -80°C until analysis. PBDE congeners were measured using solid phase extraction and GC-MS, which was operated in electron capture negative ionization (ECNI) mode as described.[24]

Behavioral Testing Paradigms: **Social Recognition Test (SRT):** Mice were allowed to habituate to a cage with two corrals (30 min.). During the 5-minute Trial 1, test subjects were presented with a corral containing a novel age- and sex-matched stimulus mouse. Social preference was tested (after a 30 min. habituation) in the subsequent 5-min Trial 2, where the test mouse was allowed to explore a now familiar mouse vs novel unfamiliar female mouse stimulus. Both stimuli mice were separately housed. **Suok:** Motor function was assessed by placing test mouse on the center of a 2m long aluminum rod suspended above ground in a dimly lit room. During the 5 min testing period, the number of falls, missteps, segments crossed, latency to leave center, grooming and directed exploration were scored live. **Forced Swim Test** (**FST):** Test mouse was placed in a clear Plexiglas apparatus containing two columns filled with water for 6 min. Time spent struggling during the last 4 min with more than one limb mobile was scored as active swimming behavior, while time spent immobile, represents depressive-like behavior. **Elevated Plus Maze (EPM):** Anxiety was measured with a black plexiglas apparatus with two open arms illuminated by overhead lights and two dimly lit closed arms. Mice were placed in center of maze and allowed to explore the apparatus for 5 min. **Olfactory Habituation/Dishabituation Test (OHT):** This test evaluates olfactory discrimination by testing a mouse's ability to habituate to and dishabituate from 2 unfamiliar social and 3 non-social scents.[25] Prior to testing, mice were acclimated for 1 hr in the testing room followed by a 45 min. acclimation to an empty (no food, water or bedding) test cage containing a neutral cotton-tipped wooden applicator as a control for novelty. Test mouse was presented with a single odor for 3 trials of 2min duration. Time spent sniffing the applicator was timed with a stopwatch. Non-social scents included: water, almond extract (1%), banana extract (1%). Banana was excluded from analysis due to lack of stimulus effect. Social scents were obtained from dirtied cages of group-housed sex-matched but stranger conspecifics. **Olfactory Preference Test (OPT):** The test mouse was acclimated in the testing room for 20 min. before being habituated in three cages for 15 min. each as described.[26] During the testing phase one of four different scents (10% peanut butter, 1% vanilla, 1% butyric acid, water) were pipetted on a 5x5cm filter paper and presented to the test subject in a randomized order for a duration of 3 min. with a 1 min. rest period between scents. **Video analysis:** All behavioral tests except Suok were scored using event-logging software (BORIS).[27] The behavioral ethogram for each test was programmed to assign keystrokes to behaviors based on predetermined criteria, which the experimenter, blind to treatment, used to score behavioral observations in the recorded videos. The duration or frequency of events assigned to a particular behavior was then measured and used to generate percent engaged in behavior out of total test time.

Quantitative polymerase chain reaction (qPCR)*: Histological Preparation*: Cryostat sections (300μM) of brain tissue were flash frozen on isopentane in OCT embedding compound and mounted. Regions of interest were ≈micro-punched out using custom made tools. Nissl staining using cresyl violet was performed on alternating

10um sections to aid in visualization of the anatomical position of the punch. *RNA Isolation:* Tissue punches were homogenized in Trizol and total RNA was prepared via a modified partial phenol-methanol extraction protocol using the RNeasy Micro Kit (Qiagen, USA). All molecular work was carried out in adherence to MIQE guidelines.[28] *Primers:* Oligonucleotide PCR primers were purchased from (Integrated DNA Technologies, Inc., USA) and were 90-110% efficient. *Oxtr* and its reference gene, *ActB*, were multiplexed using pre-designed 5' nuclease probes in the following reporter-dye combinations: the 6-Carboxyfluorescein (6-FAM) for *Oxtr*, Hexachlorofluorescein (HEX) for *ActB*; along with Zen/Iowa Black FQ double-quencher probes. For other primers, intercalating dye (SYBR Green) chemistry was used. *RT-qPCR:* was performed on a CFX Connect thermocycler with the Luna Universal or Probe one-step qPCR master mixes (New England Biolabs, USA). Four ng of RNA was used per reaction. Fold gene expression was measured relative to *ActB*, and differential gene expression was determined compared to OD using the Pfaffl method.[29]

Results: Maternal food and water intake and sex ratio of offspring were not affected, but there was a reduction in LD litter size. Body weights were elevated in HD females at sacrifice. PBDE congener accumulation in the brains of female offspring, as detected by GC/ECNI-MS, yielded two species at BDE 100 at HD and BDE 153 at LD ad HD indicating that the latter is highly retained and/or poorly metabolized. Results from a battery of behavioral tests indicate specific effects of DE-71 on anxiety, social recognition ability and olfactory discrimination of social odors. In comparison to vehicle-treated controls, exposed LD and HD female offspring showed no preference to investigate a novel conspecific over a familiar stimulus mouse previously encountered during a 30 min stimulus phase followed by a 30 min retention phase (**Fig. 1A**). No deficits were seen in exposed dams (**Fig. 1B**). OHT was used to determine if SRT deficits could be due to inability to discriminate between two different social odors. At LD, DE-71 perturbs the ability to discriminate between two social odors in females (**Fig. 1E**). Similarly, LD offspring did not show preference for the non-social odors on the olfactory preference test (OPT) relative to control (data not shown). Also, HD females showed an apparent decrease for time spent in open arm on EPM and increased latency to leave center on Suok as compared to OD indicating greater anxiety than controls (data not shown). In contrast, there was no effect of DE-71 on depressive-like behavior as measured by FST (data not shown). Interestingly, adult exposure of DE-71 did not produce alterations in the behaviors tested. In the brains of mice showing abnormal SRT and anxiety, we examined gene expression of vasopressin (*Avp*, *Avp1ar*) and oxytocin signaling (*Oxt*, *Oxtr*), which serve as markers for social recognition behavior and anxiety. In HD female offspring DE-71 significantly reduced *Avp* in paraventricular (PVN) and SON (**Fig. 1H**) and *Avp1ar* expression in SON. DE-71-induced changes in the OXTergic system were also detected such as reduced *Oxt* in LD and HD female offspring in SON (**Fig. 1G**) and increased *Oxtr* in PVN of LD females (data not shown).

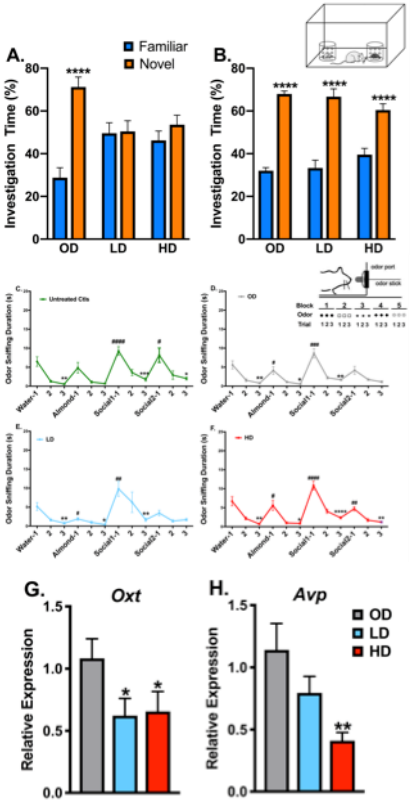

Fig.1 Effects of DE-71 exposure on female offspring and dam parameters. SRT is deficient in LD and HD female offspring (A) but not in exposed dams (B). OHT appears abnormal in LD but not HD female offspring (C-F). SON gene expression for *Oxt* are significantly reduced in LD and HD female offspring (G) and *Avp* in HD relative to *ActB* (H). Asterisks indicate statistical difference as compared to OD (A,B, G,H) or to previous odor C-F).

Discussion: Our findings using SRT indicate a deficit in social interaction and/or social memory that is critical to social hierarchy, pair bonding, maternal behavior and other social behaviors critical for species survival. Other studies examining effects of developmental exposure to BDE 47 on social interaction have reported an enhancement of mean velocity when test mice encountered a stranger mouse and less time spent with conspecifics in sociability tests indicating less interest in social interactions.[14] Another report found that perinatally exposed female offspring displayed reduced sociability.[30] In combination with these studies, our results support the possibility that early-life PBDEs can reprogram social behavior circuits. Importantly, we found that mothers of abnormal offspring that were exposed to DE-71 during adulthood did not display abnormal behaviors, indicating that organisms are more susceptible during development than during adulthood. One possible reason for deficient SRT may involve compromised olfactory processing of social cues. Indeed, LD exposed mice failed to respond to two different social odors albeit they did to non-social odors. Sham controls as well as HD exposed mice showed olfactory habituation and dishabituation typical of normal mice. In support of a PBDE effect on social olfactory discrimination, an *in vivo* study found impaired neuronal migration and dendritic development of newborn olfactory granule cells in PND 16 pups exposed perinatally to BDE 209 at 20 mg/kg via their dam mothers .[31] Evidence from human literature indicates that persons 55 to 74 years of age

exposed to a combination of PBDE congeners show normal olfactory function [32] In HD exposed female offspring augmented anxiety, which may represent increased fear of threatening social stimuli, may reduce social interest. The mechanisms of PBDE neurotoxicity in humans are still not clearly elucidated but animal studies indicate several targets including calcium homeostasis, neurotransmitter balance, nitric oxide signaling and oxidative stress.[1,15,33] We have previously demonstrated a harmful effect of acute DE-71 on evoked release of central vasopressin from the male rat SON *in vitro*.[16] In this study, we show that perinatal PBDEs reduce the expression of *Avp* and *Oxt*, gene markers of the "social" peptide neurotransmitters, vasopressin and oxytocin, respectively. Specifically, DE-71 reduces transcript levels of *Avp, Oxt* and *Avp1ar* transcripts and, in previous studies, knockout of these genes can reduce social recognition ability.[34] Importantly, LD and HD exposed females, which show SRT deficits also show reduced hypothalamic *Oxt.* Reduced hypothalamic *Oxt* may prevent oxytocin's anxiolytic effects, which may help explain the increased anxiety found in exposed female offspring. Contrary to our expectation of reduced *Oxt* in PVN, which provides excitatory OXTergic projections to olfactory nuclei, we could not implicate this target as the reason for perturbed olfaction in DE-71 exposed female offspring. Instead, in future experiments we will explore whether altered *Oxtr* expressed by olfactory nuclei may be responsible.[35] In summary, perinatal exposure to the mixture of PBDE congeners comprising DE-71 seems to reprogram forebrain neurochemistry of "social" peptides possibly leading to anti-social and anxiogenic behavior phenotypes that may be relevant to neurodevelopmental disorders.

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