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Perinatal exposure to FireMaster® 550 (FM550), brominated or organophosphate flame retardants produces sex and compound specific effects on adult Wistar rat socioemotional behavior

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Abstract

Firemaster 550 (FM550) is a flame retardant (FR) mixture which has become one of the most commonly used FRs in household items such as foam-based furniture and baby products. Because this mixture readily leaches from products, contamination of the environment and human tissues is widespread. Prior work by us and others has reported sex-specific behavioral deficits in rodents and zebrafish following early life exposure. In an effort to understand the mechanisms by which these behavioral effects occur, here we explored the effects of its constituents on behavioral outcomes previously shown to be altered by developmental FM550 exposure. The FM550 commercial mixture is composed of two brominated compounds (BFR) and two organophosphate compounds (OPFRs) at almost equivalent proportions. Both the BFR and the OPFR components are differentially metabolized and structurally distinct, but similar to known neurotoxicants. Here we examined adult Wistar rat offspring socioemotional behaviors following perinatal exposure (oral, to the Dam) to vehicle, 2000 µg/day FM550, 1000 µg/day BFR or 1000 µg/day OPFR from gestation day 0 to weaning. Beginning on postnatal day 65 offspring from all groups were subjected to a series of behavioral tasks including open field, elevated plus maze, marble burying, social interaction tests, and running wheel. Effects were exposure-, sex- and task-specific, with BFR exposure resulting in the most consistent behavioral deficits. Overall, exposed females showed more deficits compared to males across all dose groups and tasks. These findings help elucidate how different classes of flame retardants, independently and as a mixture, contribute to sex-specific behavioral effects of exposure.

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Keywords

behavior; brain; neurodevelopment; endocrine disruptors; endocrine disrupting chemicals; flame retardants

Introduction

Chemical flame retardants (FRs) are prevalent pollutants in the natural and human environment due to widespread use in myriad consumer products (Dodson et al., 2012; Ma et al., 2012; Roze et al., 2009; Stapleton et al., 2008; van der Veen and de Boer, 2012). This is concerning because human exposure to FRs has long been associated with cognitive and other behavioral deficits (Hendriks and Westerink, 2015). Multiple FR classes, most notoriously polybrominated diphenyl ethers (PDBEs), but also newer brominated FRs (BFRs) and organophosphate ester flame retardants (OPFRs), are thought to be neurotoxic and endocrine disrupting (Dishaw et al., 2014; Gaylord et al., 2020; Hendriks and Westerink, 2015) suggesting long-term risk to public health. Firemaster® 550 (FM550), containing both BFR and OPFR components, is a commonly used FR mixture and was the second most detected FR in US furniture in 2011 (Stapleton et al., 2012b). We and others have previously demonstrated in a variety of species that developmental exposure to FM550 can adversely and sex-specifically impact socioemotional behaviors including anxiety, sociality, and running wheel activity (Bailey and Levin, 2015; Baldwin et al., 2017; Gillera et al., 2019; Patisaul et al., 2013). Due to widespread BFR and OPFR use in other FR mixtures and applications, the present studies were conducted to begin to ascertain the degree to which the FM550 mixture component classes contribute to adverse outcomes on sexually dimorphic non-reproductive behaviors in rats.

FM550 comprises two brominated compounds, 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) and bis(2-ethylhexyl) 2,3,4,5-tetrabromophthalate (BEH-TEBP), previously abbreviated TBPH), triphenyl phosphate (TPHP, previously abbreviated TPP) and numerous TPHP analogs with varying degrees of aryl isopropylation (collectively called ITPs) (Phillips et al., 2017; Stapleton et al., 2014; van der Veen and de Boer, 2012). The BFR constituents, BEH-TEBP and EH-TBB, are structurally similar to bis (2-ethylhexyl) phthalate (DEHP) and its primary metabolite mono (2-ethylhexyl) phthalate (MEHP) respectively, compounds which are well-characterized endocrine disruptors via disruption of thyroid hormone action (Benjamin et al., 2017). Guideline reproductive, fertility, and developmental toxicity studies on the commercial mixture CN-2065/BZ-54, which contains only EH-TBB and BEH-TEBP, established a NOAEL of 50 mg/kg body weight per day for this mixture and, presumably, the FM550 mixture. The OPFR FM550 components, which make up approximately 54% of the mixture and structurally resemble organophosphate pesticides, are now some of the most commonly used FRs (Behl et al., 2015a). Furthermore, OPFRs are widely used as plasticizers for a variety of industrial applications (Wei et al., 2015). In particular, TPHP is a high production volume chemical commonly applied to polyvinyl chloride (PVC), circuit boards, adhesives, rubbers, and even personal care products, such as nail polish (Mendelsohn et al., 2016). Consequently, human exposure is nearly ubiquitous. While initially thought to be less toxic than the BFRs, in part because of

their more rapid metabolism and low disruptive action on acetylcholinesterase compared to their OP pesticide cousins, growing evidence suggests that presumption may be incorrect (Behl et al., 2015b; Blum et al., 2019; Doherty et al., 2019a; Glazer et al., 2018a; Zhang et al., 2019).

Especially when used as an additive, FM550 readily migrates from consumer goods (Stapleton et al., 2008; Stapleton et al., 2011; Stapleton et al., 2012b). Globally, detectable levels have been found in indoor dust, indoor/outdoor air, aquatic biota, and food, leading to widespread human exposure, primarily via ingestion and inhalation (Dodson et al., 2012; Ma et al., 2012; Roze et al., 2009; Salamova et al., 2014; Stapleton et al., 2008; van der Veen and de Boer, 2012). Their presence in aquatic environments (Ma et al., 2012; Roze et al., 2009) and air particles in the European Arctic (Salamova et al., 2014), demonstrate their environmental mobility, persistence, and bioaccumulation. Both the BFRs and the OPFRs (and their primary metabolites) are consistently detected in human tissues including breast milk, blood, hair, fingernails, and urine (Hoffman et al., 2014; Mendelsohn et al., 2016; Zhou et al., 2014). Thus, human exposure to both the brominated and organophosphate components in FM550 is ubiquitous and rising due to increased use in consumer goods (Stapleton et al., 2012a).

Developmental exposure is most concerning, with greater than 40% of breast milk samples having EH-TBB, BEH-TEBP or TPHP present (Kim et al., 2014; Tao et al., 2017). As with many chemicals, child exposure to FRs is pervasive; with children having higher exposures and body burdens than adults, including fire fighters which have some of the highest occupational exposures (Butt et al., 2014; Butt et al., 2016; Cequier et al., 2015; Fent et al., 2020; Gibson et al., 2019). Our group has repeatedly demonstrated in rats that gestational and lactational transfer of FM550 components occurs (Baldwin et al., 2017; Patisaul et al., 2013; Phillips et al., 2016). Additionally, our lab has shown placental accumulation is dose dependent and sex specific for some components (Baldwin et al., 2017). Collectively, there is compelling evidence that human FM550 exposure is widespread, likely occurring as early as prenatally, and occurs during critical periods of brain development.

Additionally, we and others have repeatedly demonstrated in multiple species, at doses far below the purported NOAEL, that developmental exposure to FM550 alters offspring behaviors, especially anxiety and exploratory behavior (Baldwin et al., 2017; Glazer et al., 2018b; Patisaul et al., 2013). As is typical for many purported EDCs, effects are often sex-specific. Most recently, we exposed prairie voles (*Microtus ochrogaster*), which display human-relevant prosocial traits including affiliation, paternal care, and social monogamy/pair bonding (McGraw and Young, 2010; Modi and Young, 2012) to 0, 500, 1000, or 2000 µg of FM550 via subcutaneous (sc) injection to the dam (oral dosing was not used because metabolism of FM550 in this species is unknown) across gestation, and then directly to the pups from birth to weaning. Exposed female offspring displayed elevated anxiety, decreased social interaction, decreased exploratory motivation, and aversion to novelty. Exposed male offspring displayed reduced sociality including failure to display a partner preference (Gillera et al., 2019). The present study builds on this collective body of work by assessing the relative contributions of the FM550 components (BFRs or OPFRs) on behavior.

Knowing that the BFRs and OPFRs are differentially metabolized and structurally distinct, we hypothesized the FM550 components would affect exploratory and socioemotional behaviors differently. To test this, female Wistar dams were orally exposed to either vehicle, 1000 µg BFR mixture (EH-TBB and BEH-TEBP), 1000 µg OPFR mixture or 2000 µg FM550 once daily from gestational day (GD) 0 to postnatal day (PND) 21. Starting on PND 65, female and male offspring went through a comprehensive battery of behavioral tests to better assess non-reproductive behaviors in both sexes. The outcomes help elucidate how different classes of FRs, independently and as a mixture, contribute to sex-specific behavioral effects of exposure.

Methods

The ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines Checklist for Reporting Animal Research was used in the construction of this manuscript with all elements met (Kilkenny et al., 2010). The ARRIVE guidelines were developed in consultation with the scientific community as part of an NC3Rs (National Centre for the Replacement Refinement and Reduction of Animals in Research) initiative to improve the standard of reporting of research using animals. (<https://www.nc3rs.org.uk/arrive-guidelines>)

Animals

Animal care, maintenance and experimental protocols met the standards of the Animal Welfare Act and the U.S. Department of Health and Human Services 'Guide for the Care and use of Laboratory Animals' and were approved by the North Carolina State University (NCSU) Institutional Animal Care and Use Committee (IACUC). All procedures were approved and monitored by a supervising veterinarian throughout the duration of the project. Wistar rats were obtained from Charles River (Raleigh, NC) and/or bred in house as indicated in humidity- and temperature-controlled rooms, each with 12-h:12-h light:dark cycles at 25°C and 45–60% average humidity in the AAALAC approved Biological Resource Facility at NCSU. As in our prior studies (Patisaul and Adewale, 2009; Rock et al., 2019), and in accordance with recommended practices for endocrine disrupting chemical (EDC) research, all rats were housed in conditions specifically designed to minimize unintended EDC exposure including use of glass water bottles with metal sippers, soy free diet (Teklad 2020, Envigo), woodchip bedding, and thoroughly washed polysulfone caging (Howdeshell et al., 2003; Li et al., 2008; Thigpen et al., 1999).

Animal Husbandry

Adult naive Wistar rats (56 females and 16 males) were obtained from Charles River (Raleigh, NC) and maintained on Teklad 2020 (Envigo) diet. All females were randomly assigned to four exposure groups with average weight per group equaling approximately 300g. Females were paired as two separate cohorts (24 and 32 females, respectively) with a male for one week. Following pairing, females were placed in clean caging and single housed for the remainder of the study. Of the 56 females, 7 failed to conceive and were therefore excluded. Additionally, 5 litters were found dead, 1 female was euthanized due to dystocia and a litter consisting of 1 pup were excluded.

Dosing

All rats were orally dosed using concentrated solutions prepared in Dr. Heather Stapleton's laboratory. Exposure concentrations were based on previous work demonstrating transplacental/lactational transfer, bioaccumulation and neurotoxicity at levels below the purported NOAEL of 50 mg/kg bw per day (Baldwin et al., 2017; Patisaul et al., 2013; Phillips et al., 2016). The single dose of FM550 was 2000 µg/day (approx. 6.6 mg/kg bw for an adult female rat) because we have previously reported behavioral differences in Wistar rats perinatally exposed to half this concentration (Baldwin et al., 2017) and a clear positive dose response in prairie voles was observed where the highest dose was 2000 µg/day by subcutaneous injection (Gillera et al., 2019). The BFR and OPFR concentrations of 1000 µg/day (approx. 3.3 mg/kg bw) were chosen to proportionally represent their concentrations in the FM550 mixture; approximately 50% BFRs and 50% OPFRs (Phillips et al., 2017; van der Veen and de Boer, 2012). Thus, BFR and OPFR exposed rats received the equivalent amount of BFR and OPFR present in FM550 mixture exposed rats. For context, following a 10-day lactational exposure to 1000 µg FM550 via the dam, concentrations of BEH-TEBP in the offspring were found to be 0.9 ± 0.2 ng/g ww with an average body burden of 27.9 ± 0.8 ng (Phillips et al., 2016). These levels are lower than reported human concentrations detected in hair and nails, 20–240 ng/g and < 17–80 ng/g respectively (Liu et al., 2015). Levels of BEH-TEBP reported in human milk reportedly range between <0.01 and 0.73 ng/g lw (Tao et al., 2017). The FM550 commercial mixture was obtained from Great Lakes Chemical (West Lafayette, Indiana) and a 50 mg/mL dosing solution was prepared by diluting the appropriate amount of FM550 into sesame oil (vehicle). The Stapleton lab also prepared the BFR and OPFR mixtures into working 50 mg/mL solutions.

The OPFR mixture included organic components of TPHP and ITPs; TPHP (Cas No. 2604051-7), 2-isopropylphenyl diphenyl phosphate (2IPPDPP; Cas No. 28108-99-8; 93925-53-2; 64532-94-1), 3-isopropylphenyl diphenyl phosphate (3IPPDPP), 4-isopropylphenyl diphenyl phosphate (4IPPDPP), 2,4-8 diisopropylphenyl diphenyl phosphate (24DIPPDPP), bis(2-isopropylphenyl) phenyl phosphate (B2IPPPP; Cas No. 69500-29-4), bis(3-isopropylphenyl) phenyl phosphate (B3IPPPP), bis(4-isopropylphenyl) phenyl phosphate (B4IPPPP), and tris(3-isopropylphenyl) phosphate (T3IPPP; Cas No. 72668-27-0). The BFR mixture contained 2-ethylhexyl-2,3,4,5- tetrabromobenzoate (EH-TBB) and bis (2-ethylhexyl)-2,3,4,5-tetrabromophthalate (BEH-TEBPA). Each dosing solution (sesame oil vehicle, BFR, OPFR and FM550) was stored in foil-wrapped scintillation vials at 4°C.

Dam exposure was once daily (~09:00AM) beginning 72 hours after pairing and continuing until postnatal day 21 (weaning). Therefore, the developmental exposure of offspring across groups (including controls) was transplacental and lactational via the dam. At the time of dosing, 20 µL of control, BFR and OPFR solution or 40µL of FM550 was pipetted onto a 1/8 of a wafer cookie (Nilla; Nabisco) resulting in the following exposure groups: 0 µg, 1000 µg BFR, 1000 µg OPFR and 2000 µg FM550. Dosing was based on average dam weight at pairing, producing exposures of approximately 3.3 mg/kg bw/day BFR or OPFR and 6.6 mg/kg bw per day FM550. Consumption was monitored to ensure that the dam ate the entire treat. During pairing, females were separated from the male and placed into clean empty cage for exposure. To minimize risk of cross contamination the administrator was not

blinded to exposure and controls were dosed first. Every other aspect of the project was conducted blinded.

Parturition and Weaning

Dam weights were collected 2 days prior to pairing and the day of separation, then once a week for three weeks after separation, and once on PNDs 1 and 21. Parturition checks were done at time of dosing (mornings) and the first day a litter was present was designated PND 0. Litter size and composition were collected on PND 1 and all culled to 12 pups with equal sex ratio when possible. To monitor for possible exposure effects on maternal care, we recorded any abnormalities in key aspects of maternal behaviors including nest building, grooming, nursing, and pup retrieval (Numan, 2006). To minimize stress on the dams, cages were visually checked every morning for presence of a defined nest, cleanliness of pups and dam, and on PNDs 1 and 10 pups were examined for evidence of milk spots and mother-pup contact (warmth of pup upon touch). Additionally, the number of pups found dead and lost litters was recorded. In total 7 females were not pregnant (1 control, 2 BFR, 3 OPFR, and 1 FM550) and 8 litters were found dead (2 control, 4 BFR, 2 OPFR, and 1 FM550). Offspring in this study were selected from the remaining 11 control, 8 BFR, 9 OPFR, and 12 FM550 litters. Weaning occurred four hours after last exposure on PND 21 and offspring were housed with same sex sibling, two per cage and given unique ear tag numbers. Tissue for future studies was collected on PNDs 10 and 90 (data to be published elsewhere). To minimize litter effects, one male and one female offspring were randomly selected from each litter to the degree possible. Because litter numbers for BFR and OPFR were below 10, to increase and equilibrate sample size across all groups, an additional (randomly selected) 3 males and females from control, BFR and OPFR groups (total, not per litter) were added. Similarly, 2 FM550 males and females were randomly selected and also added. Final numbers in behavior were as follows: controls (14F, 14M); BFR (11F, 11M), OPFR (12F, 12M) and FM550 (14F, 14M). All testing was blinded from this point.

Offspring Measurements

Weights on PNDs 1 and 10 are representative and obtained from offspring removed for future studies. To monitor growth, remaining offspring weights were collected on PNDs 21, 35, 49, 65, 80, 120, 172, 204 and day of sacrifice. Females were sacrificed on the day of estrus, and all rats were collected at approximately PND 247–261. Serum was collected from all rats via trunk blood. Wet weights were collected for adrenals, pituitaries, whole liver, kidneys, and hearts. The adrenals, pituitaries and median lobe of the livers was frozen on powdered dry ice and stored in -80°C . Using the ROUT outliers test ($Q=1\%$), one BFR male and one OPFR female were identified and removed from adrenal and heart weight analysis, respectively. Final animal numbers were as follows: controls (14F, 14M); BFR (11F, 8M); OPFR (11F, 11M); and FM550 (14F, 15M).

Serum Biochemistry

Serum biochemistry was analyzed for 14 biochemical markers of general toxicity (listed in supplemental table 1) to determine exposure effects. All procedures followed manufacturer's protocols. On day of analysis, random samples of serum were thawed at 4°C and $120\mu\text{L}$ /sample added to the Comprehensive Diagnostic profile rotors (500–7123; Abaxis, Union

City, CA). For quality assurance, a control sample (pooled from 20 random samples) was ran approximately every 25 samples. Rotors were placed in the VetScan VS2 Whole Blood chemistry analyzer and data exported. Final numbers were controls (13F,14M), BFR (11F,9M), OPFR (12F,11M) and FM550 (14F,15M). Although 21 statistical outliers from 6 biomarkers across female and male groups were identified, all data was used because no animal had greater than 2 outlying samples across all biomarkers.

Behavior

All behavioral testing was conducted at the NCSU Biological Resources Facility in rooms specifically dedicated and equipped for this purpose. Rats were housed on a partial reverse light cycle (off at 10:00 and on at 22:00) to accommodate testing during the first four hours of the dark cycle when rats are naturally most active. All testing was conducted under dim red light except for running wheel. To minimize noise or distractions, a mounted camera recorded all behavior for later analysis using TopScan (Clever Sys Inc.) software as we have done previously (Gillera et al., 2019; Rebuli et al., 2015; Rock et al., 2019) using published testing methodologies (Crawley, 2007; Insel, 1999). Prior to every test, rats were moved to the holding room to allow for acclimation. Multiple arenas were housed in one room, but males and females were never tested at the same time (except running wheels). All arenas were thoroughly cleaned between subjects with 70% ethanol.

Behavioral studies began on PND 65 and ended at PND 212. Behavioral testing was performed in a sequence considered least to most intensive for rats: open field, marble burying, sociability, social choice, running wheel, and elevated zero maze. Behavior tests occurred at least 24 hours apart. All subjects went through all tests in order, but the sequence of rats tested in each task was randomized each time. For experiments using stimulus rats, sexually naïve same sex gonadectomized juveniles (PND 40) from Charles River were used. Eight study animals were removed due to tumors (2 FM550 females and 1 OPFR male), limb injury (1 FM550 female and 1 BFR male), growth on liver (1 BFR female) and euthanasia from sickly appearance (1 FM550 male and 1 BFR male). The overall study design is shown in Figure 1.

Open field test (OF)

Testing occurred between PNDs 65 and 74. Estrus was tracked for a minimum of one week prior to testing and females were tested on the first day of diestrus. Conditions of the 30-minute open field test were described previously (Baldwin et al., 2017; Gillera et al., 2019; Hicks et al., 2016). Briefly, under dim red light (~40lux), a test animal was gently placed into the center of an empty four walled, blue opaque arena (43 cm × 58 cm × 58 cm) and allowed to freely explore for 30 min. Using TopScan software, the task floor was digitally divided into equal squares creating a 3 × 3 grid. Endpoints analyzed were number of fecal matter, distance traveled in the entire arena and center, number of entries, and time in the center. Duration in the center and total distance traveled were binned into 5-minute intervals for further analysis. Video analysis was conducted blinded and confirmed by a second, blinded observer. Three test subjects, 2F and 1M were excluded due to technical issues. Final animal numbers were as follows: controls (13F, 13M); BFR (10F, 9M); OPFR (8F,

10M); and FM550 (11F, 14M). Eight statistical outliers were removed: control (1F,1M), OPFR (3F, 1M), FM550 (1F).

Marble burying test (MB)

Marble burying occurred 3 days after open field, and rats were between PNDs 68 and 77. The task was performed as previously described for Wistar rats (Castro et al., 2017). Twelve green glass marbles (15 mm diameter, 5.2 g in weight) were uniformly distributed (4×3) in a clean standard size (26 cm $\times$ 48 cm $\times$ 20 cm) rat cage filled with 5 cm fresh, unscented rat bedding material. Test rats were gently placed in the cage facing the same corner as to not disturb the marbles. Rats could freely explore for 15 minutes and all trials were recorded for later review. During the test, the researchers remained outside the room to avoid interference. Recordings were analyzed by an observer blind to exposure groups. Marbles were considered buried when more than 2/3 of its volume was within the wood shavings. Endpoints included latency to interact with marbles and number of marbles buried at 5, 10 and 15 minutes. Four subjects were removed due to video error; OPFR (1F), BFR(1F,2M), so final numbers for analysis were controls (14F,14M), BFR (10F,9M), OPFR (11F,12M), and FM550 (14F,14M). Ten statistical outliers were removed: control (5F, 1M), BFR (2F), OPFR (1F), FM550 (1F).

Sociability

Testing occurred between PNDs 99 through 119 and all females were in diestrus. Same sex gonadectomized stimulus rats were obtained from Charles River (Raleigh, NC) and approximately PND 40 when testing began. The sociability (and social choice) test was a modified version of prior, similar studies conducted in the lab (Hicks et al., 2016; Sullivan et al., 2011). Briefly, a plexiglass arena (198.12 cm, 30.48 cm deep and 30.48 cm wide) was divided into three chambers roughly equal in size with small compartments (17.78 $\times$ 30.48 $\times$ 30.48 cm) on either side. The adjoining walls of the small compartments are perforated so the rats can interact but not harm one another. The test rat was gently placed in the center of the middle chamber and allowed to freely explore the arena for 5 minutes. A novel rat and object were placed at opposite ends of the chamber and subject interaction recorded for an additional 5 minutes. Time spent interacting with the stranger or empty cup, total investigation time, and total distance traveled, were analyzed. A sociability index was calculated by subtracting time spent with the inanimate object from time spent with the stranger rat, divided by the total time spent with both. A sociability index of 1.0 indicates 100% preference for the stranger and -1.0 indicates 100% preference for the empty cup. Due to technical issues one FM550 male video was excluded. Final numbers analyzed were controls (13F,14M), BFR (11F,9M), OPFR (12F,11M), and FM550 (12F,14M). No statistical outliers were identified.

Social Choice

Testing occurred 24 hours after sociability and subjects were between PND 100–120. The task was the same as sociability except a different same sex novel stimulus rat and a familiar (cage mate) rat were placed in the small compartments. Following five minutes of exploration, endpoints assessed including time spent with the stranger or familiar rat, total investigation time, distance travelled, and a social preference index. A social preference

index of 1.0 indicates 100% preference for the familiar rat and -1.0 indicates 100% preference for the stranger. Final numbers analyzed were controls (13F,14M), BFR (11F,9M), OPFR (12F,11M), and FM550 (12F,14M). One FM550 male was removed as a statistical outlier.

Running Wheel

Rats were age PND 105–173 on the first day of testing and female estrous stage was determined the morning of test start by lavage as previously described (Baldwin et al., 2017; Krentzel et al., 2020). Test rats were placed in a clean cage with a stainless steel 34.4 cm in diameter running wheel to analyze locomotor activity. VitalView software (Starr Life Sciences Corp, Oakmont, PA) monitored wheel running activity by counting revolutions per minute across the entire week-long period of testing via a magnetic sensor. Running wheel cages were housed in separate room from the rest of the colony. Due to limited number of wheels and space, rats were tested in cohorts of 10 or 12. One male/female representative from each exposure group was present in each cohort. Rats were placed in the wheel cages a minimum of 30 minutes prior to lights off and testing lasted one week. The first eight hours was considered a “novelty period” with exposure groups compared every hour to assess acclimation. Subsequent analysis began at the first lights on, approximately 12 hours after the wheels were introduced. Endpoints included total rotations/day, area under the curve, total rotations/6-hour bins, and slopes of activity. Final numbers were: controls (14F,14M), BFR (11F,10M), OPFR (12F,11M), and FM550 (12F,15M). Two animals were excluded because the wheels failed to record at some point during the test: Control (1M) and BFR (1M).

Elevated Zero Maze (EZM)

Subjects were aged between PNDs 180–212 and females were in estrus. Testing was conducted as previously described (Cao et al., 2013; Rebuli et al., 2015) on a 123 cm diameter circular maze consisting of 2 open sections (9.5 cm in width) and 2 closed sections (29.5 cm high walls), elevated 75.5 cm above the floor. The subject rat was gently placed into a closed region and left undisturbed for 5 minutes. All behaviors were videotaped and later analyzed to determine distance traveled, and duration/number of entries in open regions. The EZM was cleaned with 70% ethanol between subjects. Twenty-seven rats fell off the maze and thus excluded: controls (4F,4M) BFR (5F,2M), OPFR (3F,2M), FM550 (4F,3M). Final numbers were: controls (9F,8M), BFR (6F,7M), OPFR (8F,10M) and FM550 (8F,9M). Statistical outliers were: control (1M), BFR (1F), OPFR (1M) and FM550 (1F).

Statistics

The statistical approach was designed to be consistent with prior work and using published guidelines for low-dose endocrine disrupting chemical studies with sample sizes in this range (Haseman et al., 2001). Statistical analyses were performed using GraphPad Prism, version 8 (La Jolla, CA, USA). A ROUT outliers test ($Q=1\%$) was used to identify and remove statistical outliers. For all analyses, statistical significance was defined as $\alpha = 0.05$. Litter characteristics (numbers of male, female, and total pups, and male pup percentage) were compared between exposure groups and controls using Mann-Whitney tests. A Wilcoxon signed ranks test was used to test for deviation from a predicted equal sex ratio

(50%) within each exposure group. For behavioral data, first a student's one-tailed t-test comparing unexposed (control) females and males was run on all behavioral endpoints to check for known sex differences as we have done previously for similar studies (Baldwin et al., 2017; Rebuli and Patisaul, 2016). Detection of expected sex differences was considered validation that the studies were sufficiently powered to detect biologically meaningful effect sizes. Because each exposure group represents a class of fire retardant or the FM550 mixture, exposure groups were compared only to same sex controls (and not each other) using a Dunnett's test. For each outcome, effect size was calculated as recommended by multiple behavioral neuroscience groups including The American Psychological Association (Fritz et al., 2012; Lakens, 2013) and required for this journal. Effect size was calculated by Cohen's d , the effects of which are defined as small at 0.2, medium at 0.5, and large at 0.8.

Results

All supplemental material is available in the Dryad repository <https://doi.org/10.5061/dryad.4j0zpc88q>.

Litter composition

Dams weights did not differ across exposure groups at any timepoint examined (data not shown). No observable differences in the quality of maternal care were noted between exposure groups. On PND1 two nests had scattered pups (1 OPFR and 1 FM550) and one dam/pups were not fully cleaned (1 BFR) but both situations were resolved by PND 2. Overall litter size did not differ across exposure groups, but dams exposed to BFR ($U=21$, $p=0.03$, $d=0.913$) had significantly more female pups compared to controls (Figure 2). Not all pups were weighed on PND 1 or 10, only those randomly selected from each litter for future studies. Weights were analyzed via ANCOVA to take dam and litter sizes into account. No main effect was observed within sexes compared to controls nor a significant difference between unexposed males and females (Figure 3).

Subsequent offspring weights included all study rats available at that time point. Thus, because some rats were sacrificed for tissue collection, animal numbers decreased as the study progressed. A one-way ANCOVA was used to assess male and female weights while controlling for possible litter effects. As expected, control female and male weights were significantly different beginning on PND 21 and maintained throughout the study. In females there was a significant effect of exposure on PND 21 ($F_{3,148}=5.590$, $p=0.001$) and PND 35 ($F_{3,148}=4.065$, $p=0.008$). BFR ($p=0.001$, $d=0.878$), OPFR ($p=0.01$, $d=0.86$), and FM550 ($p=0.05$, $d=0.71$) exposed females were all lighter than controls on PND 21. On PND 35 only BFR females ($p=0.034$, $d=0.77$) remained lighter than controls. No effect on body weight was observed in any female exposure group post-puberty (PND 35) (Figure 4 A&B). In males, a significant effect of exposure on body weight was found at nearly all time points including PND 21 ($F_{3,140}=7.660$, $p=0.001$), PND 35 ($F_{3,140}=9.566$, $p=0.001$), PND 65 ($F_{3,140}=13.049$, $p=0.001$), PND 80 ($F_{3,139}=7.021$, $p=0.001$), PND 120 ($F_{3,63}=9.642$, $p=0.001$), PND 172 ($F_{3,68}=9.365$, $p=0.001$), PND 204 ($F_{3,69}=6.499$, $p=0.001$), and at time of sacrifice ($F_{3,68}=5.653$, $p=0.002$). PND 49 was the only timepoint no significant effect was found ($F_{3,140}=1.322$, $p=0.27$). In all cases, only the BFR and OPFR groups differed from

controls, and they were lighter at all ages, except on PND 80 when the OPFR effect did not quite reach statistical significance ($p=0.06$, $d=0.51$) (Figure 4 C&D).

Organ Weights

Relative organ weight was calculated using the following formula: (organ weight (g)/body weight of the rat on sacrifice day (g)) $\times$ 100 (Bailey et al., 2004). As previously reported, a significant sex difference was observed in liver ($t_{26}=2.414$, $p=0.01$, $d=0.91$), pituitary ($t_{26}=8.452$, $p=0.001$, $d=3.19$), adrenal ($t_{26}=7.803$, $p=0.001$, $d=2.95$), and heart ($t_{26}=2.743$, $p=0.005$, $d=1.04$) with females having higher relative organ weights (Piao et al., 2013). No organ weight effects reached significance across female exposure groups. In males, the BFR exposed rats had significantly larger livers ($t_{44}=4.886$, $p=0.001$, $d=2.20$) and pituitary glands ($t_{44}=3.242$, $p=0.01$, $d=1.43$) as well as a high effect size trend for increased heart size ($t_{44}=1.872$, $p=0.17$, $d=0.90$). Increased liver weights were also suggested in OPFR and FM550 males with both having large effect sizes ($t_{44}=2.293$, $p=0.07$, $d=0.96$ and $t_{44}=2.21$, $p=0.08$, $d=0.80$, respectively) (Supplemental Figure 1).

Serum Biochemistry

Significant sex differences in unexposed rats were observed for 7 biochemical markers, which is consistent with what has been previously reported (Chen et al., 2019; Zhou et al., 2019). Females had increased levels of albumin (ALB) ($t_{25}=9.53$, $p=0.001$, $d=3.67$), calcium (CA^{++}) ($t_{25}=3.928$, $p=0.001$, $d=1.51$), glucose (GLU) ($t_{25}=2.980$, $p=0.006$, $d=1.15$), and total protein (TP) ($t_{25}=4.504$, $p=0.001$, $d=1.73$). In contrast, male levels were higher for alkaline phosphatase (ALP) ($t_{25}=7.175$, $p=0.001$, $d=2.76$), globulin (GLOB) ($t_{25}=5.101$, $p=0.001$, $d=1.96$), and sodium ($t_{25}=2.65$, $p=0.01$, $d=1.04$). There were no statistically significant effects on any biochemical markers across female exposure groups, but in the FM550 exposed females a large effect size was suggestive of elevated ALP ($t_{46}=1.998$, $p=0.13$, $d=0.90$). In males, the effects were more pronounced. Exposure to BFR significantly elevated CA^{++} ($t_{45}=4.211$, $p=0.001$, $d=1.76$) and increased alanine aminotransferase (ALT) to a degree that nearly reached statistical significance ($t_{45}=2.361$, $p=0.06$, $d=0.74$) and OPFR males displayed increased levels of PHOS ($t_{45}=1.845$, $p=0.18$, $d=0.87$) (Supplemental Figure 2). Notably, many medium effect sizes were found and could help inform direction of future investigations (Supplemental Table 2).

Open Field

Among the control groups, no sex difference for any endpoint was identified. Because this phenomenon is maximal when females are in estrus, and for this study testing was done in diestrus, the lack of statistical significance was not unexpected. Effects of exposure were both compound and sex specific. Females exposed to FM550 had significantly decreased latency to enter the center ($t_{38}=3.476$, $p=0.003$, $d=1.41$; Figure 5B) and significantly increased defecation ($t_{38}=2.861$, $p=0.02$, $d=1.41$; Figure 5C). Similarly, a large effect size was observed for latency to enter the center ($t_{38}=2.159$, $p=0.10$, $d=0.81$; Figure 5B) in the BFR exposed females, with the BFR females entering the center earlier than controls. OPFR females also displayed a large effect size for defecation ($t_{38}=2.048$, $p=0.12$, $d=0.95$; Figure 5C). In males, defecation was significantly increased in the FM550 group ($t_{42}=2.788$, $p=0.02$, $d=1.03$; Figure 5C). There were no statistically significant effects on any other

endpoint, only trends. Large effect sizes suggested the FM550 males ($t_{42}=1.813$, $p=0.19$, $d=0.82$) spent longer in the center (Figure 5E) and BFR males tended to have greater distance travelled in the center ($t_{42}=1.749$, $p=0.21$, $d=0.87$) compared to controls (Figure 5F).

To further investigate exposure-related effects across the 30-minute session (Figure 5G), distance travelled was analyzed in 5-minute intervals. As expected, for both females ($F_{4,134}=161.8$, $p<0.001$) and males ($F_{3,113}=154.1$, $p<0.001$) there was a significant effect of time with activity decreasing over time. No significant interaction between time and exposure was found for either sex.

Marble Burying

No statistically significant sex differences were observed between control females and males for any endpoints (Figure 6). OPFR females took significantly longer to interact with the marbles ($t_{34}=2.515$, $p=0.04$, $d=1.24$; Figure 6A) and buried more marbles at 5 ($t_{34}=2.380$, $p=0.06$, $d=1.05$) and 10 ($t_{34}=2.642$, $p=0.033$, $d=1.13$) minutes of testing (Figure 6B). Marble burying was also significantly heightened in BFR exposed females at 5 ($t_{34}=3.642$, $p=0.003$, $d=2.3$), 10 ($t_{34}=3.422$, $p=0.005$, $d=1.8$) and 15 ($t_{34}=2.921$, $p=0.02$, $d=1.44$) minutes (Figure 6B). Exposure to FM550 tended to increase latency to interact with a marble in females ($t_{34}=2.017$, $p=0.13$, $d=0.90$; Figure 6A) with a large effect size but did not reach statistical significance. In males, fewer significant effects were observed. FM550 males took significantly longer to interact with the marbles ($t_{43}=5.036$, $p<0.001$, $d=1.45$), an effect that had a large effect size in the OPFR group but was nowhere close to statistical significance ($t_{43}=1.077$, $p=0.59$, $d=0.91$; Figure 6A) and had no impact on the number of marbles buried. Although the BFR exposed males buried slightly more marbles at 5 minutes ($t_{43}=1.747$, $p=0.21$, $d=0.99$), the effect was not significant (Figure 6B).

Sociability

To assess preference for a novel rat versus a novel object, a sociability index (SI) was first calculated for each animal and compared to equal preference ($SI=0$) by one-sample t-test. As expected, all groups displayed a highly significant preference for the stranger rat ($p<0.001$ for all cases; Figure 7). In the unexposed controls, males investigated the stranger more than control females ($t_{25}=2.183$, $p=0.039$, $d=0.84$; Figure 7B). Distance travelled in the three-chamber arena was also sexually dimorphic with females travelling more ($t_{25}=2.248$, $p=0.02$, $d=0.85$; Figure 7C). No significant effect of exposure was found for any endpoint. OPFR females tended to spend longer interacting with the stranger ($t_{44}=2.11$, $p=0.1$, $d=0.82$; Figure 7B), while in males, the BFR group spent less time interacting with the stranger compared to controls ($t_{44}=2.258$, $p=0.08$, $d=0.83$; Figure 7B).

Social Choice

Preference for the familiar or stranger animal was assessed by calculating a social preference index (1 = preference for the familiar rat and -1 = preference for the stranger). Control ($t_{12}=2.058$, $p=0.06$) and BFR ($t_{10}=2.092$, $p=0.06$) females tended to prefer the stranger (Figure 8A), while neither the OPFR nor the FM550 females displayed a social preference. Control ($t_{13}=3.393$, $p=0.005$), OPFR ($t_{10}=2.336$, $p=0.04$) and FM550 ($t_{12}=3.917$, $p=0.002$)

males displayed significant preference for the stranger rat. The BFR males, by contrast, displayed a slight preference for the familiar rat ($t_8=0.687$, $p=0.5$, $d=1.12$) (Figure 8A).

In females, none of the other parameters were affected by exposure (Figure 8B-E). In males, the BFR group's preference for a familiar rat was further demonstrated by the tendency to spend less time investigating the stranger ($t_{43}=1.971$, $p=0.14$, $d=0.85$), and more time investigating the familiar rat ($t_{43}=1.867$, $p=0.17$, $d=0.89$; Figure 8B). Unexposed males traveled significantly less than control females ($t_{25}=2.283$, $p=0.03$, $d=0.88$) in the 3-chamber testing arena (Figure 8E). Only the OPFR males travelled significantly more than unexposed controls ($t_{43}=2.539$, $p=0.015$, $d=0.94$) (Figure 8E).

Running Wheel

The first 8-hour period on the running wheel was analyzed independently because it was designated a habituation period. As expected, a two-way ANOVA found a significant effect of time, with both females ($F_{7,360}=12.19$, $p=.0001$) and males ($F_{7,112}=24.65$, $p=.0001$) decreasing activity (number of rotations) over time (Figure 9A). No significant interaction of time and exposure was observed. The expected large sex difference in activity (total rotations per day) was observed in the first hour of wheel activity ($t_{25}=2.511$, $p=.02$, $d=0.97$) and continued across the introductory phase and all five test days ($p=0.001$, for all 5 days), with females running significantly more than males (Figure 9B). Running wheel data beyond the introductory phase was sorted into 6-hour bins starting approximately 24 hours after rats were placed into wheel cages. Light in the animal room serves as an external cue (zeitgeber) that synchronizes the rat's activity, hence the 12:12 light:dark cycle is considered a zeitgeber period (zeitgeber (ZT) 0 is lights on and ZT 12 is lights off). To distinguish these 6-hour bins, the first six hours in the light is labelled Z0, the next six hours in light are Z6, and the night cycles are Z12 and Z18, respectively. As expected for nocturnal animals, the acrophase was during the dark cycle when rats are naturally more active (Figure 9C & D).

No statistically significant differences were observed in either female or male activity during the dark period on any day of testing (Figure 9E & F). Effect size, however, suggested all three female exposure groups, particularly the OPFR and FM550 groups, tended to have increased activity in the Z12 period beginning on day 3 and continuing throughout the study. Compared to unexposed conspecifics, the OPFR females had greater Z12 activity (medium or large effect size) on days three ($t_{45}=1.669$, $p=0.24$, $d=0.76$), four ($t_{45}=1.618$, $p=0.27$, $d=0.70$), five ($t_{45}=1.932$, $p=0.15$, $d=0.88$), and six ($t_{45}=1.4$, $p=0.38$, $d=0.61$). Similarly, FM550 females displayed greater (medium or large effect size) Z12 running on days three ($t_{45}=2.285$, $p=0.07$, $d=0.84$) and five ($t_{45}=1.120$, $p=0.56$, $d=0.55$; Figure 9E).

In the light cycle the OPFR females were significantly more active on day one Z6 ($t_{45}=2.819$, $p=0.002$, $d=1.0$), a trend that continued to day two Z6 ($t_{45}=2.257$, $p=0.08$, $d=0.91$) but not thereafter. FM550 exposed females trended higher on day two Z6 ($t_{45}=2.101$, $p=0.11$, $d=1.10$) and day 3 Z0 ($t_{45}=1.77$, $p=0.2$, $d=0.58$; Figure 9G). In males, activity in the BFR group was consistently elevated during the light period. A significant difference was found on day 1 Z6 ($t_{44}=2.95$, $p=0.01$, $d=1.16$), D2 Z6 ($t_{44}=2.924$, $p=0.02$, $d=1.30$), and day 5 Z6 ($t_{44}=2.646$, $p=0.03$, $d=1.31$). For all other periods in the light cycle, the BFR males trended more active with either a medium or large effect size. OPFR

exposure affected male activity in the light period to a lesser extent. In OPFR males a large effect was observed on day 5 Z6 ($t_{44}=2.285$, $p=0.07$, $d=1.21$), but only a medium effect on day four Z6 and nowhere close to statistical significance ($t_{44}=1.334$, $p=0.42$, $d=0.77$; Figure 9H).

Elevated Zero Maze

No sex differences on any endpoint were identified in controls, which was unexpected given that the females were in estrus and thus should have had lower anxiety and higher activity. Thus, given this plus the unexpectedly large number of rats that fell from the maze, the results must be interpreted with caution. In females, FM550 had the most pronounced effects, with FM550 females spending significantly longer exploring the open arms ($t_{25}=2.903$, $p=0.02$, $d=1.533$; Figure 10A) and making a somewhat greater number of open arm entries ($t_{25}=2.062$, $p=0.127$, $d=0.97$; Figure 10B). BFR females had a nearly significant increase in distance travelled ($t_{25}=2.467$, $p=0.06$, $d=1.513$; Figure 10C) in the open arms with a large effect size. In males, no statistically significant effects were observed but a large effect size in both the BFR ($t_{28}=1.382$, $p=0.38$, $d=0.81$) and FM550 ($t_{28}=1.778$, $p=0.2$, $d=1.04$) males suggested a lower latency to enter the open arms (Figure 10D).

Discussion

The goal of this study was to begin to discern which class of compounds in the FM550 mixture might be driving previously observed disruption of adult socioemotional behaviors following developmental exposure. This is the first rodent study to examine the full FM550 mixture alongside proportionally equivalent levels of BFR and OPFR components, on behavior. As expected, effects were exposure-, sex- and task-specific, with the most consistent set of behavioral differences found in the BFR-exposed rats. Overall, FR-exposed females showed more differences compared to males across dose groups and tasks. Although large effect sizes were detected for some outcomes, very few effects reached statistical significance, largely because of the high degree of inter-individual variability (supplemental table 3). Thus, to be fully powered for statistical significance using outbred animals requires much larger sample sizes; an outcome that is critically informative for the design of future studies. Our results begin to shed light on how the different FR classes found in the FM550 mixture differentially impact sexually dimorphic, non-reproductive behaviors.

Heightened anxiety-like behavior in BFR and FM550 developmentally exposed females

Two tests, OF and EZM, were used to evaluate anxiety-like behaviors in conjunction with aspects of locomotion and exploration (Harro, 2018). Historically, we have found anxiety to be one of the most consistently altered behaviors following developmental FM550 exposure, although which sex displays the most differences has been mixed (Baldwin et al., 2017; Gillera et al., 2019; Patisaul et al., 2013). In the present study, only females showed any evidence of altered anxiety-like behaviors, and the results across tasks were inconsistent. Of the two tasks, the data from the EZM is considered the weakest given the lack of expected sex differences in the unexposed controls and the large numbers of rats that fell from the maze. Although the decreased latency to enter the center of the OF displayed by the FM550 females suggests lower anxiety, the suggested decreases in time in the center, entries in the

center, and distance traveled in the center signify otherwise. Instead, we posit that the exposed females more quickly dart around the box in attempt to find cover, and thus enter the center earlier. Historically, we examined females in estrus because that is when their anxiety is lowest and their willingness to explore is maximal (Beatrice et al., 1994; Frye et al., 2000; Weiss et al., 1998). Here they were tested in diestrus, which likely accounts for discordance from our prior FM550 rat work (Baldwin et al., 2017; Patisaul et al., 2013). Because the primary predicted exposure-related effect is decreased center exploration, it is possible that this phenomenon can only be statistically detected in estrus when exploration of the center is maximal.

FM550 female time in the open arms of the zero maze was increased, which at first appears contradictory to their OF behavior but, as we have reported previously, rats can sometimes “freeze” in adverse environments thus confounding interpretation of this endpoint (Rock et al., 2019). Given the heightened activity in the running wheel, a phenomenon we have observed previously, we surmise that the exposed females react impulsively in the anxiety tasks and that an overall heightened level of activity plays into the fear response. This conclusion is supported by the significantly increased level of defecation the FM550 females displayed in the OF, which is also an ethologically relevant, physiological assessment of anxiety (Burke et al., 2016; Goma and Tobena, 1978; Prut and Belzung, 2003).

Overall, the data do not strongly implicate a specific FR class as driving the FM550 anxiety-related effects, but the BFR females displayed the most concordant set of behaviors in the OF and EZM. Notably, the BFR group is greatly underpowered ($n=5$) in the EZM, due to an unexpectedly large number of rats falling off the maze; a behavior that also suggests impulsivity and fear. Notably, our EZM does not have ledges, which might have helped prevent the unintentional falls (Prut and Belzung, 2003). Studies in mice have reported that OPFRs can sex-specifically alter sexually dimorphic behaviors and brain development at environmentally relevant doses comparable to those used here. In mice, offspring maternally exposed (1 mg/kg/day; GD7 to PND14) to an OPFR mixture of tris(1,3-dichloro-2-propyl), triphenyl phosphate, and tricresyl phosphate displayed altered locomotor and anxiety-related behaviors, with some outcomes sex specific (Wiersielis et al., 2020). A companion study (Adams et al., 2020) using brains from the same animals reported disrupted hypothalamic gene expression. Altered genes included peroxisome proliferator-activated receptor (PPAR γ) and estrogen receptor alpha (ER α), corroborating the hypothesis that a critical route by which OPFRs might exert detrimental effects on behavioral traits is through endocrine disruption (Gore et al., 2019). In general, the effects in females found herein corroborate previously reported heightened anxiety following developmental FM550 exposure in Wistar rats (Baldwin et al., 2017; Patisaul et al., 2013) and other species including prairie voles (Gillera et al., 2019).

Obsessive compulsive-like behaviors increased in BFR and OPFR exposed rats

The MB task is considered an ethologically relevant assessment of obsessive-compulsive disorder (OCD) related behaviors in rodents, because it is only weakly correlated with anxiety-like test outcomes, and more indicative of repetitive digging (de Brouwer and Wolmarans, 2018; Thomas et al., 2009). Additionally, the modulation of MB by serotonin

reuptake inhibitors has increased the validity of the test for OCD-like behavior (see review (Dixit et al., 2020)). The value of this test derives from the fact that human psychiatric disorders, including OCD and autism spectrum disorder (ASD), involve expression of repetitive, unwanted behaviors. Exposure to BFR and, to a lesser extent, OPFRs increased OCD-like behaviors in females with no strong evidence of altered outcomes in males other than increased latency to begin burying in the FM550 and OPFR males. It has previously been shown that MB differences between control and treated group that are detected early in the task may diminish over time in the same trial (Santini et al., 2013; Sugimoto et al., 2007). Hence, MB for each experimental rat was examined at 5, 10 and 15 minutes to assess consistency of behavioral differences across time. The BFR-exposed females had the most consistently heightened OCD-like behaviors over time.

Social behaviors minimally effected by flame retardant exposure

Very few social differences were observed in the two social tasks. In both sexes the BFR groups were less inclined to show a preference for a novel rat over a familiar one, but no other major parameters were affected. Higher levels of social anxiety can disrupt expected preference between two stimuli in a preference test (Mines et al., 2010). Furthermore, there is a bi-directional relationship between social interaction impairment and the degree of anxiety. Anxiety intensifies social impairment, and poor social function contributes to anxiety (Nicolas and Prinssen, 2006; White et al., 2010). Social differences following FM550 exposure have been reported in other species. Zebrafish exposed to FM550 0–5 days post fertilization or in adolescence (45 days post fertilization) at doses as low as 0.01 mg/L had differences in sociality (Bailey and Levin, 2015). Exposure to specific OPFR chemicals, including isopropylated phenyl phosphate, butylphenyl diphenyl phosphate, 2-ethylhexyl diphenyl phosphate and isodecyl diphenyl phosphate, led to short-term effects of altered motility in larvae, and long-term impairment of anxiety-related behavior in adults (Glazer et al., 2018a). In prairie voles, which are more prosocial than rats and mice and can form monogamous pair bonds, we found that developmental FM550 exposure dose-dependently heightened anxiety and induced social impairments in females, with males displaying milder social differences but compromised pair bonding (Gillera et al., 2019). Use of the prairie vole and other prosocial species may be more effective and appropriate for assessing the impact of chemical exposures on socioemotional behaviors.

Activity levels increased and circadian rhythms altered in BFR, OPFR and FM550 rats

Finally, the running wheel was used to obtain a comprehensive assessment of activity over days, light cycle, and the female estrous cycle. As anticipated, profound sex differences were found in the unexposed controls throughout the test, with females more active than males. In all female exposure groups, particularly the FM550 groups, heightened activity in the dark cycle trended higher but did not consistently reach statistical significance. This is concordant with our previously published study which also found that female Wistar rats exposed to FM550 displayed hyperactivity (Baldwin et al., 2017). Activity peaks were disrupted in OPFR exposed females suggesting an earlier onset of activity (Z6 in the light cycle). Disruption of male sleep patterns were observed in BFR and OPFR exposed rats. Both groups were repeatedly more active during the light period, especially right before lights off. The sleep wake cycle is tightly regulated by the circadian timing system (Pace-Schott and

Hobson, 2002). The synchronization of the circadian system is important for entraining physiological rhythms. Differences in the circadian system are linked to metabolic dysfunctions including weight gain, glucose intolerance, insulin sensitivity, and disrupted energy balance (Depner et al., 2014; Panda, 2016). Furthermore, disruption of various genes regulating circadian rhythms affect behaviors including anxiety, activity and reward (Landgraf et al., 2014). In humans, ADHD patients can have significant changes in the circadian system including shorter sleep periods (Baird et al., 2012).

Physiological and possible neuroendocrine effects of flame retardant exposure

Although the repeatable behavioral effects identified here and in prior studies indicate developmental neurotoxicity, the causal mechanisms are likely multi-factorial and remain to be identified. Evidence of thyroid hormone disruption by FM550 has been demonstrated both in vivo and in vitro (Belcher et al., 2014; Patisaul et al., 2013). In vitro, EH-TBB and BEH-TEBP interact with nuclear ERs and AR, as well as glucocorticoid and thyroid receptors (Klopcic et al., 2016; Mankidy et al., 2014; Saunders et al., 2013). TPHP and other OPFRs are known to interact with PPAR γ , gamma-Aminobutyric acid (GABA), and several nuclear receptors, including the androgen receptor (AR) in vitro (Fang et al., 2015; Gant et al., 1987; Honkakoski et al., 2004; Pillai et al., 2014). Adult zebrafish exposed to TPHP exhibited reduced fecundity, altered sex steroid levels (estradiol, testosterone, vitellogenin) and changes in gene expression related to the HPG axis (Liu et al., 2013). Thus, FM550 and its components are likely EDCs.

Effects may derive from toxicity outside the brain. We have previously reported evidence of altered endocrine, inflammatory and neurotransmitter signaling pathways in rat placentas from dams exposed to 300 or 1000 $\mu\text{g/day}$ FM550 (Rock et al., 2018). Specifically, evidence of disrupted placental serotonin production was found which is significant because disruption of placental serotonin impairs fetal brain development (Bonnin and Levitt, 2011; Velasquez et al., 2013) resulting in profound neurochemical and behavioral changes (Deneris and Gaspar, 2018; Olivier, 2015; Versteeg et al., 2015). We posit the OPFRs may be primarily driving this effect because we have shown, in Wistar rats, prenatal exposure disrupts the organization of serotonergic pathways in the fetal forebrain and placental tryptophan metabolism (Rock et al., 2020). These findings suggest that brain and behavioral outcomes may derive from effects at the level of the placenta. Finally, it is possible that FR exposure may have impacted maternal behavior and, consequently, offspring behavior. Although no study to date has examined the possible effects of FM550 or its components on maternal behaviors, exposure to estrogenic EDCs during gestation/lactation or developmentally can affect the maternal brain and parental behavior (Catanese et al., 2015; Cummings et al., 2010; Keller et al., 2019). All dams were monitored for key aspects of maternal behavior including nest building, grooming, pup interaction and lactation and no apparent changes were observed. Much more work is needed to elucidate the underlying neural, endocrine, and other pathways affected by developmental exposure to FM550 or its BFR and OPFR components as they relate to socioemotional behaviors including anxiety and overall activity.

Finally, offspring relative organ weights are informative for identifying other potential targets of FR toxicity, most notably in liver, pituitary and heart. We have previously reported increased FM550-related fibrosis in developmentally exposed male Wistar rats (Patisaul et al., 2013). In the present study, multiple biochemical markers were sporadically elevated across groups, possibly indicating early onset of either liver or kidney disease (Supplemental Table 1). These data are largely concordant with a prior study showing that a 90 day exposure to 2, 20, 70 or 140 mg/kg day ITP mixture in adult rats (~PND60) tended to cause reduced body weight in females, induce histological changes in liver (including weight), disrupt activity of xenobiotic-metabolizing enzymes, induce hypertrophy of the adrenal glands, and increase lipid accumulation in the adrenal cortex (Wade et al., 2019).

Conclusion

Here we demonstrated the potential for developmental exposure to FM550 and its component classes (BFRs and OPFRs), at environmentally relevant levels, to impact socioemotional behaviors. Relevance to humans is demonstrated by emerging work associating exposure to FM550 or its components to cognitive and memory-related deficits in children (Castorina et al., 2017a; Castorina et al., 2017b; Doherty et al., 2019b; Luo et al., 2020). The potential for significant, unexpected and long-term human exposure is illustrated by the presence of a sofa containing FM550 causing EH-TBB levels to be three times higher in household dust compared to levels in a household containing a sofa without FM550 (Hammel et al., 2017). Although developmental exposure most likely occurs from contaminated household dust, occupational exposure to FRs is also significant and particularly high for firefighters (Estill et al., 2020; Fent et al., 2020). As the use of FR chemicals continues to increase, so does the risk of developmental exposure in humans. Both BFR and OPFR urinary metabolites are repeatedly found in children, yet the extent of potential adverse effects remains to be fully identified. Neither BFR nor OPFR exposed rats consistently replicated FM550-related outcomes thus neither class appears to singly account for the effects of the full mixture. This may be due to differences in accumulation or metabolism when given alone, a possibility requiring further exploration to fully understand human risk.

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Highlights

- Flame retardants (FRs) are ubiquitous in our environment and suspected neurotoxins.
- Developmental exposure to the mixture Firemaster 550 (FM550) disrupts behavior.
- FM550 contains brominated and organophosphate components.
- How those component classes contribute to adverse behavioral phenotypes was our focus.
- Rats were exposed via the dam orally through gestation and lactation.
- Effects were exposure-, sex- and task-specific.
- The brominated compounds produced the most consistent behavioral deficits.
- Females showed more deficits compared to males.
- These findings help elucidate how different FR classes contribute to behavioral deficits.

Figure 1:
Study design

Figure 2:

Litter outcomes. Number of total, male and female pups per litter. Dams exposed to BFR had significantly more females compared to controls. Graphs depict mean $\pm$ SEM (*p 0.05).

Figure 3:

Representative offspring weights of PND1 (A) and PND 10 (B) from pups randomly selected for future studies. No main effect of exposure was observed for either sex nor a significant sex difference between unexposed males and females. Graphs depict mean $\pm$ SEM.

Figure 4:

Offspring weights. As expected, control female and male weights were significantly different beginning on PND 21 and maintained throughout the study. All exposed females weighed significantly less than controls on PND21 and only BFR females weighed less on PND 35 (A&B). Males exposed to either BFR or OPFR weighed significantly less than controls at all timepoints except PND 49 and PND 80 for OPFR only (C&D). Graphs depict mean $\pm$ SEM (*p 0.05, **p 0.01, ***p 0.001).

Figure 5:

Effects of developmental exposure to FM550 or components on open field behavior. (A). Females exposed to FM550 or BFR had decreased latency to enter the center (B). FM550 exposed animals had significantly more defecation compared to controls. Both BFR and ITP exposed animals had medium or large effect sizes (C). No effects on distance travelled, time in the center, or center entries were observed in either sex (D, E, F). Distance travelled in all groups decreased overtime as expected with no exposure effects (G). Graphs depict mean $\pm$ SEM (*p 0.05, **p 0.01).

Figure 6:

Effects of developmental exposure to FM550 or components on marble burying. OPFR females and FM550 males took significantly longer to interact with marbles. FM550 females tended to have a longer latency to interact with the marbles (A). The BFR and OPFR females buried significantly more marbles than controls (B). Graphs depict mean $\pm$ SEM (*p 0.05, **p 0.01, ***p 0.001).

Figure 7:

Effects of developmental exposure to FM550 or its components on social behavior. In all groups, test animals displayed a highly significant preference for the stranger animal vs. the object (A). Unexposed males spent significantly longer (B) and travelled less than (C) females. Graphs depict mean $\pm$ SEM (** $p < 0.001$). Significant sex differences indicated by Ψ ($\Psi < 0.05$).

Figure 8:

Developmental exposure to FM550 and components effects on social choice behavior. The social preference for female controls and BFR exposed animals approached significant preference for the stranger animal while the OPFR and FM550 females had no preference. Unexposed, OPFR and FM550 males preferred the stranger animal (A). In females, no other parameters were affected by exposure (B–E). In the males, the BFR exposed males tended to spend less time with stranger and more with familiar but it was not statistically significant (B). Unexposed males travelled significantly less than control females in the 3-chamber testing arena, and OPFR males travelled further (E). Graphs depict mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$) Significant sex difference indicated by Ψ ($\Psi < 0.05$).

Figure 9:

Developmental exposure to FM550 and components effects on running wheel activity. All female and male groups habituated to the running wheel across the introductory period (A). Additionally, females consistently ran significantly more than males during the introductory period and across all 5 days (A & B). As expected for nocturnal animals, the acrophase was during the night cycle when they are naturally more active (C & D). No statistically significant differences were observed in either female or male activity during the dark period, on any day of testing (E & F). However, based on effect size, all three female exposure groups, particularly the OPFR and FM 550 groups, tended to have increased activity in the Z12 period beginning on day 3 and continuing throughout the study (E). In the light cycle the OPFR females were significantly more active on day 1 Z6 and FM550 trended higher on D2 Z6 and D3 Z0 (G). In males, activity in the BFR group was consistently elevated during the light period and to a lesser extent, OPFR exposure were affected in the light period (H). Zeitgeber (ZT) 0 is lights on and ZT 12 is lights off. To distinguish these 6-hour bins, the first 6 h in the light is labelled Z0, the next 6 h in light are Z6, and the night cycles are Z12 and Z18, respectively. Graphs depict mean $\pm$ SEM (*p 0.05). Significant sex difference is indicated by Ψ (Ψ = 0.05).

Figure 10:

Developmental exposure to FM550 and its components on elevated zero maze. In females, FM550 had the most pronounced effects, with FM550 females spending significantly longer exploring the open arms (A) but the somewhat greater number of open arm entries was not statistically significant (B). BFR females had a nearly significant increase in distance travelled (C) in the open arms with a large effect size. In males, no statistically significant effects were observed but a large effect size in both the BFR and FM550 males suggested a lower latency to enter the open arms (D). Graphs depict mean $\pm$ SEM (* $p < 0.05$).