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Sex-Specific Enhanced Behavioral Toxicity Induced by Maternal Exposure to a Mixture of Low Dose Endocrine-Disrupting Chemicals

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Abstract

Humans are increasingly and consistently exposed to a variety of endocrine disrupting chemicals (EDCs), chemicals that have been linked to neurobehavioral disorders such as ADHD and autism. Many of such EDCs have been shown to adversely influence brain mesocorticolimbic systems raising the potential for cumulative toxicity. As such, understanding the effects of developmental exposure to mixtures of EDCs is critical to public health protection. Consequently, this study compared the effects of a mixture of four EDCs to their effects alone to examine potential for enhanced toxicity, using behavioral domains and paradigms known to be mediated by mesocorticolimbic circuits (Fixed Interval (FI) schedule controlled behavior, novel object recognition memory and locomotor activity) in offspring of pregnant mice that had been exposed to vehicle or relatively low doses of four EDCs, Atrazine (ATR – 10mg/kg), Perfluorooctanoic acid (PFOA – 0.1 mg/kg), Bisphenol-A (BPA - 50µg/kg), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD – 0.25µg/kg) alone or combined in a mixture (MIX), from gestational day 7 until weaning. EDC-treated males maintained significantly higher horizontal activity levels across 3 testing sessions, indicative of delayed habituation, whereas no effects were found in females. Statistically significant effects of MIX were seen in males, but not females, in the form of increased FI response rates, in contrast to reductions in response rate with ATR, BPA and TCDD, and reduced short term memory in the novel object recognition paradigm. MIX also reversed the typically lower neophobia levels of males compared to females. With respect to individual EDCs, TCDD produced notable increases in FI response rates in females, and PFOA significantly increased ambulatory locomotor activity in males. Collectively, these findings show the potential for enhanced behavioral effects of EDC mixtures in males and underscore the need for animal studies to more fully investigate mixtures, including chemicals that converge on common physiological

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substrates to examine potential mechanisms of toxicity with full dose effect curves to assist in interpretations of relevant mechanisms.

Introduction

Multiple classes of chemicals, e.g. fertilizers and herbicides, plastics, organic pollutants, metals, flame retardants and heat stabilizers, have been shown to have endocrine disrupting characteristics. Given the chemical heterogeneity of EDCs, a broad range of physiological targets have been identified. EDCs may interfere with the production, secretion, transportation, metabolism, binding action and/or excretion of natural hormones (Diamanti-Kandarakis et al., 2009). There is mounting evidence that developmental exposures to EDCs impact neurochemical pathways leading to lifelong disease susceptibility and behavioral deficits into adulthood (de Cock et al., 2012, Schantz and Widholm, 2001, Schug et al., 2011). Developmental EDC exposures can cause physiological reprogramming of hormonal homeostasis with impacts on peripheral and neurological hormone relationships, e.g., glucocorticoids and glutamate, estrogen and dopamine function (Patisaul and Adewale, 2009, Vandenberg et al., 2012). EDCs have been implicated in the etiopathogenesis of ADHD, autism, and other neurodevelopmental and behavioral disorders (de Cock, Maas, 2012, Schug, Janesick, 2011); thus, understanding the consequences of developmental exposure to low dose EDC mixtures for neurological disease etiology is vital (Colborn, 2004b, de Cock, Maas, 2012).

Studies in animal models indicate that monoaminergic neural pathways are specifically altered as a result of developmental exposure to EDCs, particularly mesocorticolimbic dopaminergic systems (Palanza et al., 2008). For instance, in vivo and in vitro studies indicate atrazine (ATR) causes a reduction in striatal dopamine (Coban and Filipov, 2007, Hossain and Filipov, 2008). In rats, prenatal exposure to ATR decreased striatal dopamine and decreased locomotor activity (Bardullas et al., 2011, Lin et al., 2013a, Rodríguez et al., 2012). Prenatal exposures to perfluorooctanoic acids (PFOAs), as fire retardants, increased home-cage activity in male mice, and enhanced astrogliosis and pro-inflammatory cytokines in the hippocampus and cortex of rats (Onishchenko et al., 2011, Zeng et al., 2011). Mice exposed to low dose TCDD in the perinatal period exhibited hypo-activation of the prefrontal cortex, increased brain monoamines and increased social behavior abnormalities (Ahmed, 2011, Endo et al., 2012). In mice, prenatal bisphenol A (BPA) exposure altered the development of central dopaminergic systems and resulted in hyperactivity and increased reward-seeking behavior (Mizuo et al., 2004a, Mizuo et al., 2004b, Narita et al., 2006, Suzuki et al., 2003). Also, prenatal and developmental exposure to BPA has been shown to elicit multiple sex-specific behavioral deficits including increase impulsivity, neophobia and exploratory behavior, altered maternal behavior and adult social behavior (Adriani et al., 2003, Gioiosa et al., 2013, Palanza et al., 2002, Patisaul et al., 2012, Spulber et al., 2014, Wolstenholme et al., 2011).

This is notable given that mesocorticolimbic dopamine systems mediate multiple behavioral domains particularly related to cognitive and executive functions as well as memory consolidation, temporal discrimination, exploratory and food-reinforced reward behaviors

(Cory-Slechta et al., 1997, Rossato et al., 2013, Sy et al., 2010). Further, there is growing appreciation that maternal and early life exposure to environmental toxicants can potentially have a profound lifelong impact on the central nervous system (CNS). Evidence is accumulating that these alterations occur at low doses, as many EDCs show non-monotonic dose-response relationships (Vandenberg, Colborn, 2012). However, most of this information has been obtained on a chemical-by-chemical basis. The neuroendocrine and behavioral deficits associated with EDC mixtures, which is more representative of human exposures, have not been evaluated (Diamanti-Kandarakis, Bourguignon, 2009).

There has been significant controversy as to whether low levels of EDCs can act together, particularly when present at lower than threshold concentrations, particularly if they have different mechanisms of action. However, as we have previously pointed out, multiple insults occurring concurrently at multiple sites within the e.g., dopamine system, may constrict the range and flexibility of compensatory mechanisms, thereby compromising integrity and viability of the system, and ultimately be more damaging than multiple insults at the same molecular target sites (Cory-Slechta, 2005). Indeed, recent studies of low dose mixtures of chemicals reducing androgens via different mechanisms resulted in additive male reproductive dysfunction effects (Howdeshell et al., 2008, Rider et al., 2009).

Based on this presumption, this study sought to determine whether the impact of multiple EDCs, all known to impact brain mesocorticolimbic systems but by different mechanisms, would yield enhanced effects in combination, as manifest in behaviors known to be mediated by these dopamine/glutamate circuits and correspondingly whether observed effects would, as expected, differ by sex. To this end, we assessed performance under the fixed-interval schedule of reinforcement, object exploration, novel object recognition, and spontaneous locomotor activity, all behaviors in which mesocorticolimbic system function is important (Cory-Slechta, Pazmino, 1997, Rossato, Radiske, 2013, Sequeira-Cordero et al., 2013), in offspring exposed developmentally to four EDCs alone and combined in a mixture. As a first such study pursuing this hypothesis, it did not include full concentration-effect curves for all EDCs but was intended to provide critical information that could be used to more specifically formulate subsequent studies.

Methods

Developmental Exposure

C57BL/6 mice (age 9 weeks) were obtained from the Jackson Laboratory (Bar Harbor, ME). Nulliparous females were housed with males, and checked daily for presence of a vaginal plug. The day a vaginal plug was found was designated as gestational day (GD) 0. Pregnant mice were then individually housed for the remainder of the study. Pregnant mice were exposed orally to either the single EDC dose or the combination of all four doses: Atrazine (ATR – 10mg/kg), Perfluorooctanoic acid (PFOA – 0.1 mg/kg), Bisphenol-A (BPA - 50µg/kg), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD – 0.25µg/kg) and their mixture (MIX), from GD 7, a time point chosen because it represents the period shortly after embryonic implantation, until weaning (Wang and Dey, 2006). Vehicle (VEH) control dams were gavaged with peanut oil containing an equivalent concentration of anisole and given control treats daily. These compounds have all been shown to influence mesocorticolimbic and

neuroendocrine systems (see above), and doses were defined as relatively low doses either based on current human reference doses or were below levels typically shown to cause effects in animal studies (Fenton et al., 2009, Rowe et al., 2008, Vom Saal and Hughes, 2005, Vorderstrasse et al., 2006). ATR and BPA (Sigma Aldrich, St. Louis, MO) were dissolved in peanut oil and administered to pregnant mice daily via puffed wheat cereal. PFOA (Sigma Aldrich, St. Louis, MO) was dissolved in water and administered daily via puffed wheat cereal. TCDD (Cambridge Isotopes, Cambridge, MA) was dissolved in anisole and diluted in peanut oil. Female mice were administered TCDD via oral gavage on GD 7 and 14, and postnatal day (PND) 2, as TCDD has a relatively longer half-life (11 days), this schedule facilitates an even dosing throughout the pregnancy (Gasiewicz et al., 1983). MIX exposed mice were given puffed wheat cereal containing the same dose of each EDC and gavaged on the same schedule as each singly treated group. Random observations of dams indicated total consumption of the treated puffed wheat cereal used for dosing.

Offspring were weaned at PND 21. In order to minimize litter specific effects, no more than 2 mice per sex per dam were used. All mice were pair-housed in microisolator cages after weaning in a specific pathogen-free facility under a 12hr light-dark cycle maintained at 22 ± 2 °C at the University of Rochester Medical Center. All animal treatments were conducted with approval of the Institutional Animal Care and Use Committees at the University of Rochester.

Maternal Health and Weight

Pregnancies were monitored daily to evaluate whether there were differences in time to parturition, litter size, or sex ratio of all the offspring born to each dam. As adults, all offspring were weighed to ensure that there were no significant body weight differences before behavioral testing began. In preparation for behavioral testing, mice were food-restricted to 85% free feed weight. For this purpose, mice were fed individualized amounts of food and weighed five times a week to ensure maintenance of individual 85% free feeding weights throughout behavioral testing.

Behavioral Battery

Behavioral testing of offspring began at 60 days of age, and consisted of locomotor activity, followed by novel object recognition performance, and finally performance on a fixed interval schedule of food reward, all behaviors under control of brain mesocorticolimbic systems. These specific behaviors were chosen to provide information on potential changes in cognitive and motor related functions. Since performance on the FI schedule has been demonstrated to influence subsequent behavioral performances on reinforcement schedules, it was tested last in the sequence.

Locomotor activity—Locomotor activity was assessed in photobeam chambers to determine any treatment-related motor deficits that might also impact performance on the novel object recognition paradigm or Fixed Interval performance. Spontaneous locomotor activity was measured in chambers (27.3 cm × 27.3 cm × 20.3 cm) equipped with 48-channel infrared photobeams (Med Associates Inc., St. Albans, Vermont). Photobeam breaks were recorded every 5 minutes for an hour to assess horizontal, vertical, and

ambulatory movements. Mice were initially habituated to the locomotor activity chambers in two 60 min sessions occurring on consecutive days and locomotor behavior assessed during the third session. Ambulatory counts were defined as the number of beam breaks while in ambulatory movement. Vertical activity was defined as total time spent breaking any photobeam in the Z-axis, with the z-axis measured by photobeams mounted 7 cm above the floor of the locomotor box. Horizontal counts were defined as the number of beam breaks in a 2×2 inch photobeam box that were non-ambulatory (i.e. movement that occurred while the animal remained within the 2×2 inch defined photobeam box). Resting time was defined as time spent with no new photobeam breaks.

Novel Object Exploration and Recognition—NOR testing consisted of two phases and was conducted in an open plexiglass arena (dimensions: 30.5 cm × 30.5 cm × 30.5 cm). In the first session, mice were placed in the test environment that contained two objects for 10 min, enabling individual assessments of response to novelty, decreased exploratory behavior or neophobia. During that time, side preference, exploration time, and patterns of exploration among treatment groups were assessed.

The second session of the NOR paradigm assessed short-term memory, premised on an animal's awareness of novelty and its memory of already familiar objects. In the second session, occurring 24 hours after session 1, mice were returned for 5 min to the arena, in which a novel object now replaced one of the previous two objects. Placement of the novel object was counterbalanced across treatments to preclude bias. All sessions were videotaped and scored by a reviewer blinded to treatment group. Exploration was defined as a mouse oriented towards the object with head first entry into a pre-marked 2 cm circle surrounding the object. A recognition index was calculated based on the time spent with novel object compared to the familiar object (time spent with novel object/(time spent with novel object + time spent with familiar object). Time per approach was calculated by the average time spent per bout of investigation for either the novel or familiar object.

Fixed-interval schedule of reinforcement—The fixed interval (FI) schedule of reward is a behavioral paradigm that can be used to assess multiple behavioral dimensions, including learning, ability to inhibit responding and motivation to act for reward. FI testing occurred in sound-attenuated operant chambers (30.5 cm × 24.5 cm × 21 cm; Med Associates Inc., St. Albans, Vermont). Chambers were equipped with a grid floor, speaker, house light and three response levers connected to a feeder that delivered 20 mg food pellets. Only one lever was activated for reinforcement contingencies during the FI schedule, although responses on the other two levers were recorded. Mice were trained to depress the response levers for food reward using a previously described protocol (Cory-Slechta et al. 1985). The training schedule consisted of a 6 hour variable time 60 sec fixed ratio 1 (VT60FR1) schedule followed by 1 hour FR1 sessions across several days. Training was considered complete when subjects earned 50 rewards within a FR1 session. Subjects required between 1 and 4 FR1 sessions, and numbers of sessions to meet this criterion did not differ by treatment group.

A 60 second FI schedule was then imposed in the next behavioral test session. Sessions were initiated by the first response which initiated a 60 second fixed interval. The first lever press

response after completion of the 60 sec interval produced food reward and initiated the next 60 second fixed interval. Sessions were 30 minutes in duration and carried out for 6 sessions to focus on early acquisition of characteristic FI performance. Standard FI performance was measured by overall response rate (total number of responses on the designated FI lever divided by total session time) evaluating the integrated performance on the schedule that includes both responding and pausing between rewards. Additionally, post-reinforcement pause (PRP) time and inter-response times (IRT) were measured during each FI session to assess behavioral mechanisms associated with any observed differences in overall response rate. PRP is defined as the amount of time between food reward delivery and the first lever press response in the next 60 sec interval. IRT is median time-lapsed between FI responses. These two measures allow separation between the time delay to begin lever pressing from the duration of time between lever presses once lever pressing has resumed.

Statistical Analysis

Breeding outcomes and NOR data were analyzed by ANOVA with treatment group as a between groups factor. Adult weight, FI and locomotor testing were analyzed separately using a repeated measures ANOVA with treatment group (ATR, TCDD, PFOA, BPA, MIX and VEH) as a between group factor and session number as a continuous within group factor. Post hoc testing was conducted contingent on ANOVA outcomes using Tukey-Kramer HSD to correct for multiple comparisons for one-way ANOVAs and contrast tests for repeated measures ANOVAs. Given that the two sexes characteristically maintain different hormone profiles and show sex-specific behavioral responses to EDC exposures (Bigsby et al., 1999, Gioiosa et al., 2007, Jašarević et al., 2013), and that our previous data indicates robust sex specific differences during these behavioral tests (Allen et al., 2014, Weston et al., 2014), all analyses were conducted separately by sex. Statistical analyses were conducted using JMP Pro 9.0 (SAS Institute Inc., Cary, N.C.).

Results

Breeding outcomes

There were no treatment related deficits observed during pregnancy or time to parturition. Litter sizes (Table 1, $F(5, 32) = 0.16$, $p = 0.98$) and sex ratios (F / M) were equal across treatment groups (Table 1, $F(5, 32) = 0.57$, $p = 0.73$). There were no significant weight differences in pups during the month before behavioral testing began (females: $F(5, 40) = 1.44$, $p = 0.23$; males: $F(5, 65) = 1.31$, $p = 0.27$).

Behavioral Testing

Locomotor Behavior—Significant EDC-induced changes in locomotor activity were seen only in males (Fig. 1). Males in nearly all EDC groups (ATR, TCDD, PFOA and MIX) showed significantly increased horizontal movement across the three days of locomotor assessment ($F(5,68) = 3.93$, $p = 0.004$) relative to vehicle control. Interestingly, these effects emerged only with repeated testing, with significant differences seen only during the second and third test sessions, findings consistent with a potential reduction in environmental habituation (Session 1: $F(5,68) = 1.05$, $p = 0.39$; Session 2: $F(5,68) = 3.42$, $p = 0.008$; Session 3: $F(5,68) = 2.62$, $p = 0.03$). In addition to the elevated horizontal activity, PFOA

males also exhibited decreased resting time (Resting: $F(5,68) = 2.38$, $p = 0.048$) and increased ambulatory movements in session one of the locomotor assessment ($F(5,68) = 2.71$, $p = 0.03$). No significant treatment-related differences were found for vertical activity in males.

Novel Object Exploration and Recognition—A robust decrease in the rate of exploration during the initial phase of NOR testing (session 1) occurred in all treatment groups in both sexes. Specifically, males and females exposed to BPA, PFOA, TCDD, ATR and MIX all showed reductions in initial exploration of the novel objects, as indicated by significant reductions in time spent per approach (Figure 2: male by treatment group: $F(5, 62) = 13.13$, $p < 0.001$, post hoc: Tukey-Kramer HSD: ATR, $p = 0.001$, BPA, $p < 0.006$, PFOA, $p < 0.005$, TCDD, $p < 0.001$, MIX, $p < 0.001$; female by treatment group: $F(5, 40) = 8.22$, $p < 0.001$, post hoc: Tukey-Kramer HSD: ATR, $p = 0.002$, BPA, $p < 0.001$, PFOA, $p < 0.001$, TCDD, $p < 0.001$, MIX, $p = 0.03$). However, while EDC-treated females compensated by increasing the number of times they approached the novel objects, EDC-treated males did not (Figure 2: male by treatment group: $F(5, 62) = 1.00$, $p = 0.43$; female by treatment group: $F(5, 40) = 4.31$, $p < 0.01$). Consequently, only EDC-treated males showed significantly decreased overall exploration time, suggesting the different treatments resulted in deficits in exploratory behavior in males (Figure 2: male: $F(5, 62) = 9.60$, $p < 0.001$, post hoc: Tukey-Kramer HSD: ATR, $p < 0.001$, BPA, $p < 0.001$, PFOA, $p = 0.09$, TCDD, $p < 0.001$, MIX, $p < 0.001$; female: $F(5, 40) = 0.95$, $p = 0.45$).

In accordance with other studies using NOR testing (Aubele et al., 2008a, Frick and Gresack, 2003), control males had significantly longer exploration times than did control (vehicle) females (Figure 3: VEH male: VEH female, $t = 2.41$, $p < 0.05$). However, MIX exposed males actually showed significantly lower rates of exploration compared to MIX exposed females (Figure 3: MIX male: MIX female, $t = -3.40$, $p < 0.05$), suggesting a reversal of sex-associated differences in exploratory behavior by the MIX treatment. The reversal of this sex specific exploration bias suggests that MIX males experienced the greatest reduction of exploratory behavior, as female rates did not change significantly in response to MIX treatment (Figure 3: VEH male: MIX male, $t = 4.42$; $p < 0.001$; VEH female: MIX female, $t = -0.08$; $p = 0.93$). This enhanced reversal of sex specific exploratory behavior was only seen in the MIX treatment group (data not shown).

In the second session, enhanced deficits in short-term memory were observed in MIX exposed males, as reflected in the significantly reduced recognition indices based on overall exploration time (Figure 4, $F(5, 61) = 2.92$, $p < 0.02$; Tukey-Kramer HSD: $p = 0.018$). Males exposed to single EDCs did not show significant differences in recognition time compared to control (Figure 4, $F(5, 61) = 2.92$, $p < 0.02$; Tukey-Kramer HSD: ATR, $p = 0.48$; BPA, $p = 0.10$; TCDD, $p = 0.47$; PFOA, $p = 0.26$). For females, significant treatment-related deficits in short-term memory were not observed based on the overall exploration time recognition index (Figure 4: $F(5, 40) = 0.84$, $p = 0.52$). However, TCDD-treated females spent significantly less time per each approach of the novel object compared to VEH exposed females, suggesting slight memory deficits ($t = 2.02$, $p = 0.02$), as VEH females spent more time with the novel object during each exploration bout (data not shown).

Fixed Interval Reinforcement Schedule-Controlled Behavior—Enhanced behavioral deficits were seen in male MIX offspring during the acquisition of the FI reinforcement schedule (Figure 5). MIX males showed significantly increased FI response rates, suggesting an inability to inhibit responding or impulsive responding (treatment (TX) by session, $F(25, 310) = 3.08$, $p < 0.001$, post hoc: mixture vs. control, $F(5, 310) = 4.43$, $p < 0.001$). This behavioral response was unique to MIX exposed males, as increased rates of responding were not seen in any other male treated group. In fact, ATR, BPA and TCDD males exhibited significant reductions in response rates relative to controls, suggestive of reduced attention to task or impaired motivation (post hoc: ATR vs. control, $F(5, 310) = 3.86$, $p < 0.002$; TCDD: $F(5, 58) = 5.61$, $p < 0.001$; BPA: $F(5, 310) = 5.61$, $p < 0.001$). Response rates of PFOA exposed males did not differ from control males ($F(5, 310) = 0.68$, $p = 0.64$).

TCDD exposed females showed marked increases in FI response rates during early sessions, suggesting potential increased impulsivity or inability to acquire the temporal pattern of response (Figure 5; TX*session, $F(5, 39) = 2.38$, $p < 0.001$, post hoc: TCDD vs. control, $F(5, 195) = 8.38$, $p < 0.001$). MIX females did not show enhanced effects during the FI schedule (post hoc: MIX vs. control, $F(5, 195) = 1.45$, $p = 0.21$). In fact, the response rates of MIX females were significantly lower than TCDD exposed females (post hoc: MIX vs. TCDD, $F(6, 195) = 2.91$, $p < 0.02$).

PRP time was not significantly different between treatment groups or treatment groups over time, for either males or females (Figure 5: males: TX; $F(5, 62) = 0.95$, $p = 0.45$; TX by session, $F(25, 310) = 1.19$, $p = 0.17$; Figure 5: females: TX; $F(5, 39) = 0.62$, $p = 0.68$; TX by session, $F(25, 195) = 0.57$, $p = 0.95$). Consistent with the significantly increased overall response rates in MIX exposed males and TCDD exposed females, however, both also exhibited significantly reduced IRT times. Collectively with the absence of changes in PRP times, these findings suggest an inability to inhibit responding and not a more rapid initiation of responding within the interval, i.e., a temporal discrimination dysfunction, likely explained increased overall response rates of MIX males and TCDD females (Figure 5: males: TX by session, $F(25, 310) = 2.05$, $p < 0.01$, post hoc: MIX vs. controls, $F(5, 310) = 19.41$, $p < 0.001$; Figure 5: females: post hoc: TCDD vs. controls, $F(5, 195) = 3.22$, $p < 0.01$). However, male treatment groups that showed significant decreases in overall response rate, i.e., ATR, BPA and TCDD, did not show significant alterations in either PRP time or in IRT values, suggesting that modest but non-statistically significant delays to begin responding in the interval and lack of repeated responding once initiated may combine to create significantly lower overall response rates.

Discussion

Developmental exposure to EDCs alone and as a mixture led to multiple behavioral deficiencies in adulthood in behaviors that included a fixed interval reinforcement schedule (FI), novel object recognition (NOR) paradigm and spontaneous locomotor activity, all known to involve mesocorticolimbic mediation (Antunes and Biala, 2012, Cory-Slechta, Pazmino, 1997, Cory-Slechta et al., 1998, Ennaceur et al., 2005, Evans and Cory-Slechta, 2000) predicated on the fact that developmental exposures to these EDCs are known to alter

monoaminergic neural pathways (De Coster and van Larebeke, 2012, Frye et al., 2012, Suzuki, Mizuo, 2003), particularly along the mesolimbic dopaminergic systems (Palanza et al. 2008). Specifically, behavioral deficits were found in response to all five EDC treatments, but were enhanced with exposure to a mixture of EDCs in a sex-specific manner, with only males showing unique behavioral deficits compared to the single EDC exposed males.

MIX exposed males had significantly elevated FI rates of responding, whereas components of the MIX, including ATR, TCDD and BPA significantly reduced overall rates of responding on the FI schedule, demonstrating that the combination produced a unique alteration of adult male FI performance. It is interesting to think about the possibility that these results reflect altered underlying mesocorticolimbic dopamine function, as we have previously reported a U-shaped curve relating dopamine to FI response rates (Cory-Slechta, 1998), wherein either increases or decreases in dopamine ultimately suppress FI response rates through their ability to suppress locomotor (low levels) and induce stereotypy (high levels). This will require future assessment of dose effect curves for dopamine-related changes in response to the single EDCs and the mixture. However, the dichotomous nature of the effects is notable given the accompanying disparate interpretations, i.e., that MIX could lead to increased impulsivity or an inability to inhibit responding and/or time discrimination deficits, whereas individual components induce a lack of attention to task and/or even impaired motivation or impaired temporal discrimination. Considerations of non-linearity of underlying neurotransmitter mediating functions will be critical to interpretations of behavioral outcomes in future studies of mixtures and may indicate that disparate behavioral changes can nevertheless reflect interactions in a common neurotransmitter system.

Male specific MIX effects likewise characterized novel object recognition performance. Evidence of neophobia for both sexes exposed developmentally to EDCs occurred during the initial session of the NOR task in that all EDC groups of both sexes showed decreased time per approach to objects relative to vehicles, but only males exhibited significantly decreased overall time spent exploring the objects, suggesting a more severe effect in males. These findings may be consistent with the EDC profile of locomotor activity and habituation failure changes in response to EDCs in males, who failed to exhibit the typical decreases in horizontal movement over repeated activity testing sessions in the locomotor chambers, suggesting an absence of habituation to the novelty of the chamber. These differences were not seen in female mice.

Novelty exploration and neophobia is typically a sexually dimorphic behavior. Male preference for novelty has been suggested to have adaptive functions (Wilson and Daly, 1985), with support from rodent studies indicating that males spend more time exploring novel objects than females (Cyrenne and Brown, 2011, Frick and Gresack, 2003). Even when females show increased exploratory behavior, developmental exposure to EDCs eliminates any sex specific differences in exploratory behavior and alters sex-specific neurochemistry (Gioiosa, Fissore, 2007, Rubin et al., 2006). The current findings add further support to those assertions that developmental exposure to EDCs creates sex-specific alterations in exploratory behavior (Patisaul, Sullivan, 2012). In this study, vehicle males

showed significantly increased overall exploration time with objects compared to vehicle females, findings consistent with prior reports (Cummings et al., 2013, Cyrenne and Brown, 2011, Frick and Gresack, 2003, Wilson and Daly, 1985). In contrast, mice developmentally exposed to the mixture of EDCs showed the opposite relationship, as MIX exposed males showed significantly decreased exploration times compared to MIX exposed females. There is experimental evidence that such behavioral differences can be hormonally mediated, as rats exposed to a long-acting gonadotrophin-releasing hormone antagonist, Antide, demonstrated a similar behavioral reversal. Antide treated males showed reduced serum testosterone and diminished preference for novelty compared to females (Cyrenne and Brown, 2011). Additionally, gonadectomized males showed diminished exploratory behavior compared to control males, while gonadectomized males given testosterone increased novel object exploration (Aubele et al., 2008b). Future studies will assist in understanding whether these sex-dependent EDC effects relate to feminized hormone physiology and/or altered neurochemical function.

Novel object recognition is considered an assay of short-term memory. Memory deficits occur after exposure to EDCs, e.g., BPA, ATR and TCDD, and have been associated with spatial and object memory deficits in developmentally exposed male mice (Belloni et al., 2011, Brouillette and Quirion, 2008, Lin et al., 2013b, Palanza, Gioiosa, 2008, Tian et al., 2010, Xu et al., 2011). However, in the current study after controlling for multiple comparisons, only MIX males showed significant memory deficits with a reduced recognition index in the NOR assay based on overall exploration time of novel relative to familiar objects. The unique influence of the mixture of EDCs on male memory warrants further investigation as the reduced exploration patterns during the first NOR test session may be responsible for the memory deficits seen during the recognition phase of the NOR testings, as animals that have reduced experience with the objects during that habituation phase may have difficulty separating novel from familiar in the recognition phase. As such, it is critical to include information from the NOR training sessions in any analyses of short-term memory.

Of additional note in this study were the dramatic behavioral consequences of TCDD exposure in females. As TCDD is a selective AhR receptor, it is relevant that AhR genes, ARNT, and ARNT2, are sensitive to perinatal steroid hormone manipulations and expressed in sexually dimorphic brain regions (Petersen et al., 2006) such as the sexually dimorphic nuclei of the preoptic area, which contain estrogen receptors, and the anteroventral periventricular nucleus, which is a structure critical for sex-specific, estrogen-dependent, luteinizing hormone surge release (Petersen and Barraclough, 1989, Petersen et al., 1989, Petersen, Krishnan, 2006). Gene expression for AhR, ARNT, and ARNT2, mRNAs was also found in the sexually dimorphic medial preoptic regions and the ventromedial hypothalamus, brain regions critical to expression of male sexual behaviors (Gray and Brooks, 1984, Meisel and Sachs, 1994) and female (Kow and Pfaff, 1998). Of further note is the report that nearly all AhR gene expression in the brain of developing and adult rats is found in GABAergic neurons (i.e., those expressing the glutamic acid decarboxylase, the enzymes critical for GABA synthesis) suggesting GABA inhibitory function may be a target of TCDD exposure (Hays et al., 2002) which could modulate the dopamine/glutamate balance of mesocorticolimbic systems (Harte and O'Connor, 2005, Yamaguchi et al., 2011).

Animal and human studies suggest that exposure to TCDD elicits memory deficits, with some suggestion that females are more susceptible than males (Barrett et al., 2001, Brouillette and Quirion, 2008).

PFOA exposed males also showed increased ambulatory movement and decreased resting time, suggesting significantly increased hyperactivity compared to controls. Multiple studies also identify a causal role of developmental exposure to PFOA with increased hyperactivity in rodents, and epidemiological studies support a role for PFOA in human ADHD etiology, particularly at low exposure levels (Hoffman et al., 2010, Johansson et al., 2008, Ode et al., 2014, Onishchenko, Fischer, 2011). Taken together, the role of low level EDC exposures, particularly to perfluorinated chemicals, in the etiology of attention-related behaviors warrants further investigation.

Some limitations to the current study should be noted. One is the fact that TCDD was administered differently (gavage) than BPA, ATR and PFOA. It has become increasingly clear that prenatal stress can act synergistically to enhance behavioral and neurochemical toxicity (Cory-Slechta et al., 2010, Weiss and Bellinger, 2006). Because TCDD has a longer half-life, TCDD is traditionally administered via oral gavage: as a result TCDD, mixture and control mice were all gavaged once every seven days, potentially exposing these groups to increased prenatal stress, as gavage has been long known to cause stress (Brown et al., 2000). Therefore, prenatal stress is a potential confounding variable for MIX, TCDD and control mice. Future research should identify alternative means of appropriate administration of TCDD to reduce stress or should specifically investigate the potential role prenatal stress may have on synergistically eliciting neurobehavioral deficits associated with EDC exposure. The fact that control mice did receive vehicle gavage, however, provides support for the enhanced behavioral toxicity of MIX relative to control values.

In summary, the sex dependent alterations observed in behavioral performance, e.g., impulsivity and neophobia, could correspond with sex-biased human behavioral disorders, like addiction and ADHD. Further, this male-biased susceptibility to EDC mixtures was observed in the absence of signs of overt toxicity, or changes in body weight, litter size or sex ratios, and despite the heterogeneity of the chemical classes and associated mechanisms. Collectively, these findings support the need to further evaluate relationships between developmental exposure to EDCs and sex dependent neurobehavioral toxicity. EDCs have been implicated in the increased incidence of neurobehavioral disorders with sex biased prevalence rates, such as ADHD and autism, where males are increasingly susceptible (Colborn, 2004a, b, Frye, Bo, 2012). Such research will should include the generation of full dose-effect curves for individual EDCs and the MIX, particularly given findings of non-monotonic dose effect curves and the recognition of the non-linearity of underlying neurobiological substrates, e.g., dopamine (Cory-Slechta, 2005, Cory-Slechta, O'Mara, 1998, Evans and Cory-Slechta, 2000). Further, the fundamental role of sex hormones in behavioral neurodevelopment, including time-course assessments of hormone profiles will be requisite to this understanding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- All males exposed to EDCs showed exploratory deficits during NOR habituation.
- Only MIX exposed males showed a sex specific reversal in exploratory behavior.
- MIX exposed males displayed reduced object recognition during NOR testing.
- MIX exposed males showed uniquely elevated response rates during FI acquisition.
- TCDD exposure produced notable increases in FI response rates in females.

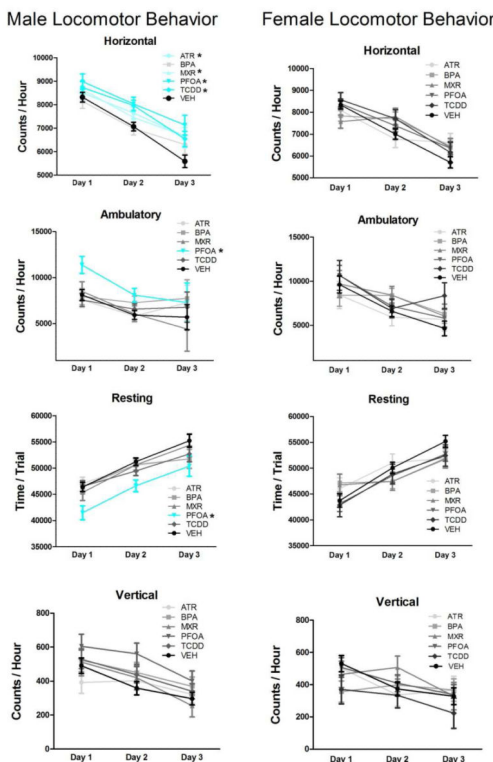
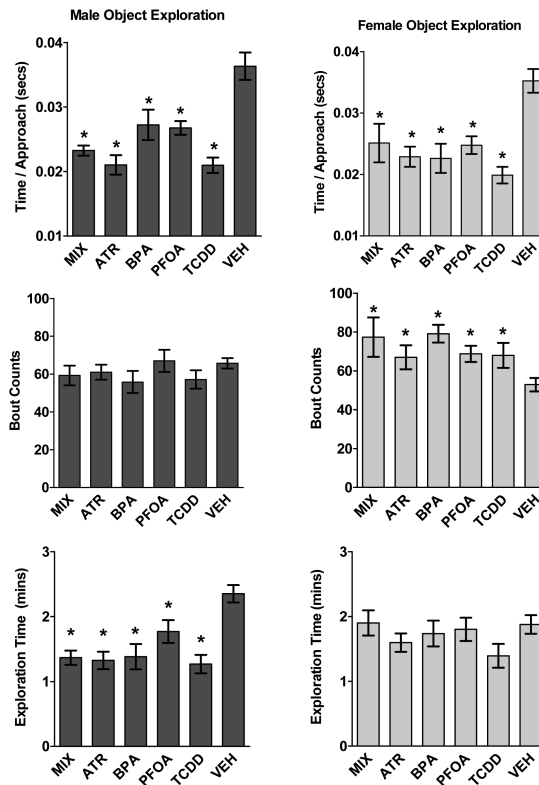


Figure 1a-h. Locomotor behavior was significantly altered in the exposed male offspring but not females. Group mean \pm S.E locomotor counts and time for males (left) and females (right) for treatment groups as indicated. Asterisks indicate significant difference from vehicle control. Males: MIX = 7, ATR = 11, BPA = 10, PFOA = 9, TCDD = 14, VEH = 22; Females: MIX = 6, ATR = 6, BPA = 9, PFOA = 12, TCDD = 5, VEH = 16.

**Figure 2a-f.**

NOR exploratory behavior was disrupted by EDC exposure. Group mean \pm S.E measures of NOR performance in the first testing session, including time spent per each approach to the object replaced in session 1 by a novel object, number of bouts of approach to that object, and time spent exploring the object for males (left column) and females (right column) for treatment groups as labeled. Asterisks signify significant difference from vehicle control. Males: MIX = 7, ATR = 11, BPA = 10, PFOA = 8, TCDD = 14, VEH = 17; Females: MIX = 5, ATR = 6, BPA = 7, PFOA = 9, TCDD = 5, VEH = 15.

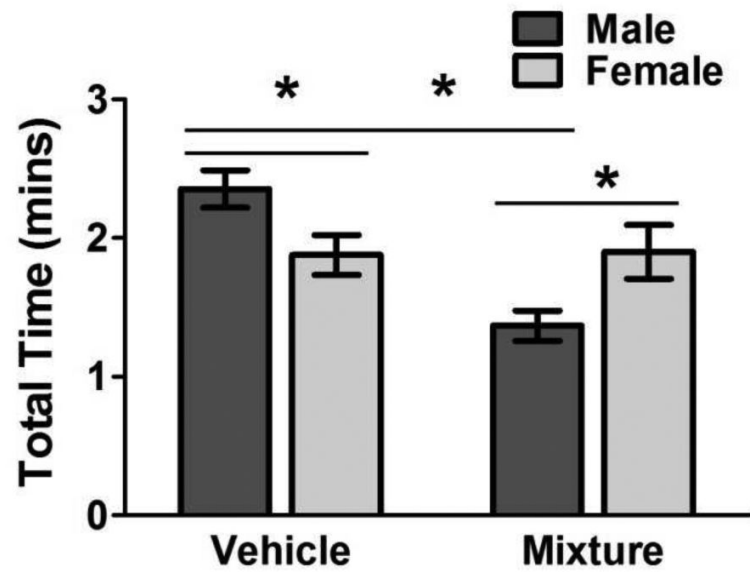


Figure 3. Reversal of sexually dimorphic exploratory behavior was seen in mixture group. Group mean \pm S.E total time (min) exploring the objects in session 1 for male and female, vehicle and mixture-treated groups as indicated. Asterisks designate significant differences between groups indicated by horizontal lines. Males: MIX = 7, ATR = 11, BPA = 10, PFOA = 8, TCDD = 14, VEH = 17; Females: MIX = 5, ATR = 6, BPA = 7, PFOA = 9, TCDD = 5, VEH = 15.

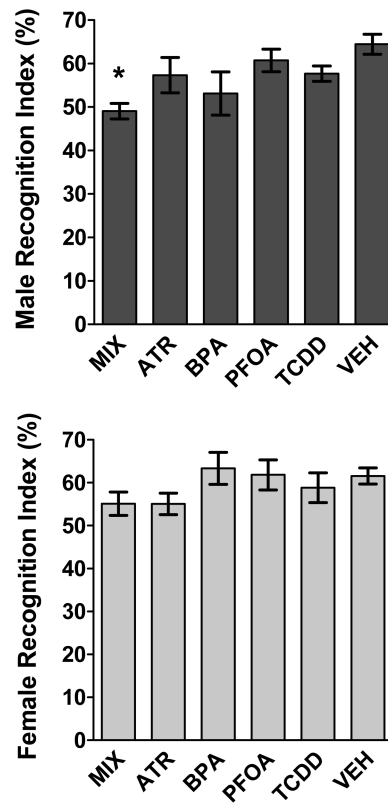


Figure 4a and b.

Enhanced NOR recognition deficits in MIX exposed male mice. Group mean \pm S.E recognition index values (percent) in session 2 for males (a, top) and females (b, bottom) for treatment groups as indicated. Asterisks indicate significant difference from vehicle control. Males: MIX = 7, ATR = 11, BPA = 10, PFOA = 8, TCDD = 14, VEH = 17; Females: MIX = 5, ATR = 6, BPA = 7, PFOA = 9, TCDD = 5, VEH = 15.

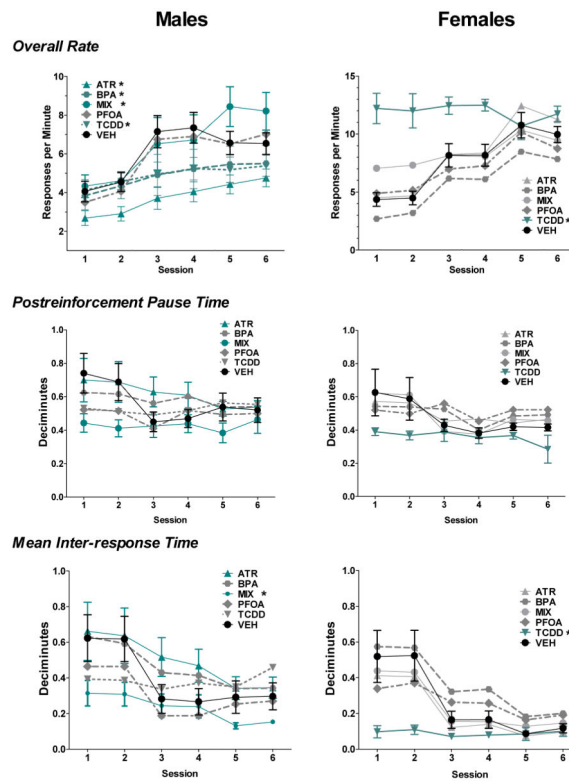


Figure 5a-f.

Enhance deficits in FI behavior seen only in male mice exposed to the mixture. Group mean \pm S.E. changes in overall response rate (top row), postreinforcement pause time (middle row) and mean inter-response times of male (left column) and female (right column) treatment groups as indicated across the sessions of FI testing. Asterisks next to the designation of a treatment group designate significant difference from control. Males: MIX = 7, ATR = 11, BPA = 10, PFOA = 9, TCDD = 14, VEH = 17; Females: MIX = 4, ATR = 6, BPA = 7, PFOA = 9, TCDD = 5, VEH = 15.

Table 1

Pregnancy Outcomes and Adult Weights

Treatment	Total number of Dams	Mean litter size \pm S.E.	Average sex ratio \pm S.E. f/m	Mean Adult Weight \pm S.E. (F)	Mean Adult Weight \pm S.E. (M)
ATR	7	7.4 \pm 0.24	0.81 \pm 0.13	18.9 \pm 0.32	23.8 \pm 0.38
BPA	6	7.2 \pm 0.71	1.12 \pm 0.17	19.5 \pm 0.31	23.5 \pm 0.40
MIX	6	6.6 \pm 1.02	0.98 \pm 0.37	19.2 \pm 0.36	24.8 \pm 0.48
PFOA	6	7.5 \pm 0.28	1.24 \pm 0.28	18.6 \pm 0.26	24.0 \pm 0.43
TCDD	7	7.0 \pm 0.19	0.65 \pm 0.18	18.9 \pm 0.36	24.2 \pm 0.33
VEH	11	7.0 \pm 0.76	1.16 \pm 0.35	19.0 \pm 0.20	23.7 \pm 0.29

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