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Enduring autism-like phenotypes and deregulated hypothalamic prosocial peptides after early-life exposure to indoor flame retardants in male C57BL/6 mice

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Abstract

Polybrominated diphenyl ethers (PBDEs) are neuroendocrine-disrupting chemicals that produce adverse neurodevelopmental effects. PBDEs have been implicated as risk factors for autism spectrum disorder (ASD), which is characterized by abnormal psychosocial functioning and is commonly comorbid with cognitive deficits and sensory abnormalities. Here, we used a mouse model with translationally relevant exposure to establish direct causal evidence that maternal transfer of a commercial mixture of PBDEs, DE-71, produces ASD-relevant behavioral and neuroendocrine deficits in male offspring. C57Bl6/N mouse dams were exposed to a commercial PBDE mixture, DE-71, via oral administration of 0 (vehicle control, VEH/CON), 0.1 (L-DE-71), or 0.4 (H-DE-71) mg/kg b.w./day for 10 weeks, spanning from 3 weeks prior to gestation through the end of lactation at postnatal day (PND) 21. Mass

spectrometric analysis indicated a dose-dependent transfer of PBDEs (in ppb) to brains of F1 male offspring at PND 30, with reduction in levels by PND 110. DE-71-exposed adult F1 male offspring displayed ASD-relevant abnormal neurobehavioral phenotypes, including impaired social novelty preference (SNP) despite intact general sociability and exaggerated repetitive behavior. There were also milder effects on long-term social recognition memory (SRM). DE-71-exposed mice also displayed altered olfactory discrimination of social odors without altered odor preference for non-social odors, impaired novel object recognition memory, and reduced open field habituation relative to VEH/CON. However, no changes were observed in sensorimotor, anxiety-, or depressive-like behaviors. At the molecular level, DE-71-exposed males displayed deregulated gene markers of prosocial neuropeptides, oxytocin, vasopressin, and PACAP systems in hypothalamic and forebrain regions. *Oxt* was upregulated in the paraventricular nucleus (PVN); *Avp* was upregulated in the PVN and bed nucleus of the stria terminalis (BNST) but downregulated in the lateral septum (LS); *Avp1ar* and *Adcyap1* were upregulated in the BNST; and *Adcyap1r1* was upregulated in the PVN, supraoptic nucleus (SON), and BNST. Peripheral OXT was increased in L-DE-71 males, while no changes in plasma AVP were observed. These findings demonstrate that developmental PBDE exposure produces enduring behavioral and neuroendocrine phenotypes that resemble core domains of ASD, which may result from early neurodevelopmental reprogramming within central social and memory networks.

KEYWORDS
developmental origins of health and disease (DOHaD), endocrine-disrupting chemicals, neurodevelopmental disorder, organohalogen, social brain network

1 | INTRODUCTION

Autism spectrum disorder (ASD) is a clinically heterogeneous neurodevelopmental disorder (NDD) diagnosed based on deficits across two core symptom domains: social communication and interaction, and restricted, repetitive patterns of behavior, interests, or activities.[1,2] ASD is frequently accompanied by comorbidities such as intellectual disability, attention deficit hyperactivity disorder (ADHD), and olfactory impairments.[3,4] According to the US Centers for Disease Control (CDC), based on 2022 data, the prevalence of ASD has increased fivefold since 2000 and currently affects 1 in 31 children, with a disproportionately male bias (male-to-female ratio 3.4:1).[5] This epidemic-like rise in ASD prevalence cannot be entirely explained by changes in diagnostic criteria and improved detection and awareness.[6] While ASD has strong genetic underpinnings, genetic factors only contribute an estimated 40%–80% of ASD heritability.[7] Moreover, findings from monozygotic twin studies show that genetics alone cannot account for ASD risk, suggesting a role for environmental factors.[8] Thus, the etiology of ASD is hypothesized to involve gene–environment (G × E) interactions, with environmental factors exacerbating genetic susceptibility[9]; however, the complex interactions between toxicants and genetic factors remain understudied, highlighting a critical gap in understanding their contribution to ASD risk.

Mounting evidence shows significant associations between early-life exposure to certain endocrine-disrupting chemicals (EDCs) and NDD risk.[10,11] This includes polybrominated diphenyl ethers (PBDEs), a class of persistent organic pollutants (POPs) used in a wide range of consumer products including building materials, electronics, textiles, plastics, and foams.[12] PBDEs have been detected in human serum, placenta, and breastmilk and are readily transferred to the developing fetus, contributing to their neurodevelopmental toxicity.[13] PBDEs have been banned or voluntarily phased out, leading to slow but measurable declines in the concentration of some congeners in environmental and biota samples.[14,15] However, PBDEs continue to leach into ecosystems due to their lipophilicity and poor degradation and bioaccumulate in human and wildlife tissues via inhalation, oral and dermal exposure.[16–18] PBDE contamination is predicted to remain an ongoing health problem for decades to come due to their long half-lives, persistence in e-waste,[19] and their inadvertent reappearance into the environment and consumer products via recycling and microplastic vectoring.[20]

Findings in humans and animals have raised concerns about PBDE exposure in the framework of the developmental origins of health and disease (DOHaD) hypothesis, suggesting that PBDE exposure during early developmental windows of biological plasticity may

disproportionately disrupt neurobehavioral outcomes.[21,22] Epidemiological studies have found that developmental PBDE exposure is associated with poor executive function, lower IQ, attention difficulties, and disrupted behavioral regulation, reported as impulsivity and reduced social competence, suggesting that PBDEs may negatively impact children's social development.[23–27] Recently, findings from the HOME study also revealed that male adolescents whose mothers had an increased gestational serum $\sum_5$BDE concentration exhibited decreased social competence.[28] Postnatal BDE-47 exposure was associated with a significantly higher risk of poor social competence symptoms in a cohort of 4 year olds from Spain.[24] Similarly, in the EARLI study, elevated maternal BDE-47 was associated with reduced social reciprocity scores (SRS) in their children.[29] However, data from the HOME longitudinal cohort provide inconclusive evidence for ASD risk by PBDEs since serum levels of PBDEs in pregnant mothers (2003–2006) have been associated with both greater (BDE-28) and fewer (BDE-85) autistic behaviors in their toddlers.[25]

Experimental studies in murine models have provided supporting evidence that specific PBDE congeners adversely affect locomotor behavior, learning, and memory in offspring.[13,30,31] However, data about the negative impact of PBDEs on psycho-social behavior remain limited. Broader investigations of brominated flame retardants (BFR) reported disrupted social cognition across several ASD-relevant domains,[32–34] although other studies found no significant effects. These inconsistencies may be explained by the absence of a second stressor or "hit," the type of BFR studied, developmental timing of exposure, sex, species and/or exposure model.[33–36] The preclusion of studying multi-congener mixtures has also been recognized as a possible reason for the lack of detection of an association between POPs such as PBDEs and social deficits. We recently reported that F1 female progeny perinatally exposed to the penta-PBDE mixture, DE-71, display ASD-relevant behavioral characteristics concomitant with deregulated markers for hypothalamic prosocial genes[37]; however, whether exposure in male offspring produces similar effects was not studied.

Oxytocin (OXT) and vasopressin (AVP) are neuropeptides produced in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamic neurohypophysial system (HNS) where they play essential roles across multiple social cognition domains such as social memory, social/emotional recognition, and social reward.[38–41] Both neuropeptides can be altered in autistic individuals[42–44] and also used in the treatment of psychosocial disorders.[45,46] Evidence from animal models shows that thyroid hormones (THs) regulate hypothalamic OXT/AVP mRNA and content,[47,48] and axonal release.[47–50] We and others have also shown that the rat and the mouse neuroendocrine PVN and SON are vulnerable to disruption by PBDEs and other EDCs with TH-disrupting properties, in parallel with the display of social behavior deficits.[37,51–56] PBDEs, along with structurally related polychlorinated biphenyls (PCBs), such as those found in Aroclor 1254, reduce physiologically stimulated AVP release from SON (and PVN) magnocellular neuroendocrine cells.[51,52] This suggests that PBDEs may contribute to ASD-like traits by affecting the central release of AVP and/or OXT from either somatodendritic compartments[57] and/or axonal collaterals/projections of AVP- and OXT-ergic neurons to extrahypothalamic social centers.[58] Taken together, such PBDE effects on the HNS and social behavior may, in part, be mediated through PBDE-induced TH disruption. Indeed, much evidence exists on the endocrine disruption of thyroid (and reproductive) endocrine systems inflicted by PBDEs.[13,59] However, further research is needed to shed light on these interrelated processes as potential contributors to PBDE neurodevelopmental outcomes.

To the best of our knowledge, no comprehensive characterizations of ASD neurobehavioral phenotypes exist using murine models of developmental exposure to BDE mixtures or single congeners. Studying the mechanisms of action of the former is particularly warranted since there are reports of synergistic and antagonistic effects of individual congener constituents in PBDE mixtures that are not predictable as a result of exposure to individual congeners. Studies of these mechanisms, particularly those involving sexually dimorphic neuroendocrine pathways, could inform on the safety of substitute and emerging flame retardants and other halogenated EDCs that are not regulated. In particular, the PBDE mixture, DE-71, recapitulates the congener profile historically measured in North American populations. We, therefore, conducted a study which comprehensively tested the effects of maternal transfer of DE-71 on the core ASD domains and constructs that represent comorbidities in ASD related to cognition, mood, and sensory deficits in offspring.

Since ASD affects each sex differently and PBDE congeners show estrogenic, anti-estrogenic, and/or androgenic actions,[60] it is important to characterize the effects of PBDEs in each sex. The present study builds on our prior work in female offspring to extend the investigation in male mice.[37] Here, we exposed mouse dams to the commercial PBDE mixture, DE-71, at low doses designed to model chronic, low-level exposure to humanly relevant congeners. We show that perinatal exposure to DE-71 leads to permanent impairments in social recognition and general memory, repetitive behavior, olfactory function, and prosocial neuromolecular markers in brain regions involved in regulating complex social behaviors in male mice. Some, but not all outcomes, were similar to those we reported in female offspring. Our findings emphasize the importance of considering sex as a biological variable in studies of environmental neurotoxicity and implicate PBDEs as contributors to ASD-related neurodevelopmental outcomes.

2 | METHODS

2.1 | Animal housing and care

C57Bl/6N mice were generated using breeders purchased from Charles River Labs (West Sacramento, CA). Mice were housed 2–4 per cage in standard static polycarbonate plastic cages containing corn-cob bedding in a specific pathogen-free vivarium maintained on a 12:12-h light: dark cycle, controlled temperature (21.1–22.8°C) and a relative humidity of 20%–70%. Mice were allowed ad libitum access to rodent chow (LabDiet 5001; Laboratory Diets, USA) and tap water from glass bottles with metal sippers. It should be noted that phytoestrogens and other potential EDCs (e.g., mycotoxins) in the rodent diet and/or corn cob

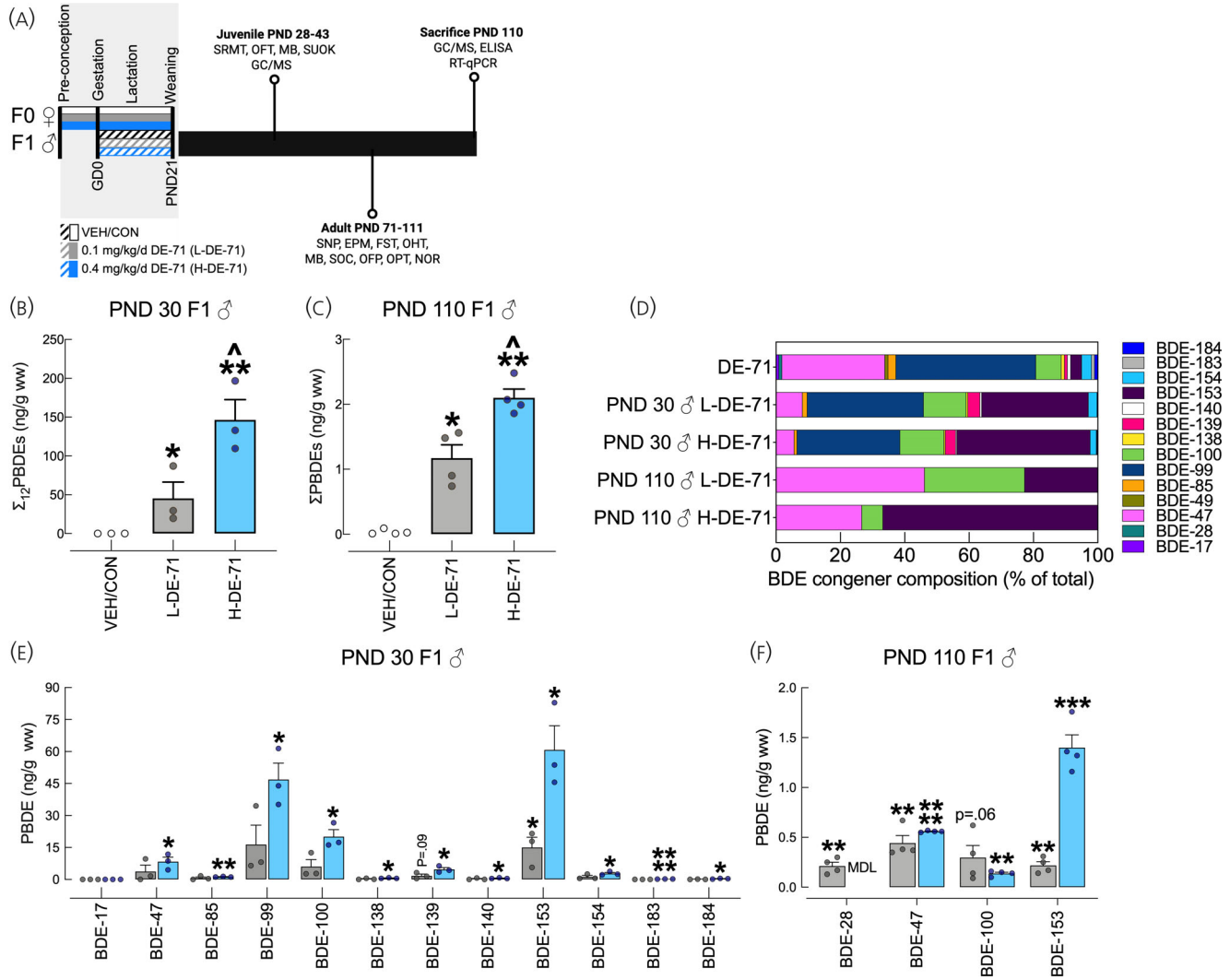

FIGURE 1 Maternal dosing paradigm for DE-71 produces persistent BDE congener penetration in male F1 offspring brain. (A) Direct exposure to DE-71 in adult dams (F0♂; solid shading), began ~3–4 weeks pre-conception and continued during lactation until pup weaning at PND 21. Indirect exposure in male offspring (F1♂; hatched shading) occurred perinatally (GD 0 to PND 21). Offspring were subjected to behavior tests at different ages as shown. (B) The sum concentrations of the 12 PBDE congeners ($\sum_{12}$PBDEs) in ng/g wet weight (w.w.) detected at PND 30. (C) The sum concentrations of the 3–4 PBDE congeners ($\sum$PBDEs) detected in ng/g w.w. at PND 110. (D) BDE composition (% total) in DE-71 and in offspring brain samples. The 7 congeners that comprise <1% of DE-71 were displayed as 1% for better distinction. (E, F) Absolute congener concentrations at PND 30 and 110. *Statistical difference compared to VEH/CON, *p < .05, **p < .01, ***p < .001, ****p < .0001; ^Statistical difference compared to L-DE-71, ^p < .05. n, 3–4 individuals/group. EPM, elevated plus maze; ELISA, enzyme-linked immunosorbent assay; FST, forced swim test; GD, gestational day; MB, marble burying; NOR, novel object recognition; OFT, open field test; OHT, olfactory habituation/dishabituation test; OPT, olfactory preference test; PND, postnatal day; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SNP, social novelty preference test; SRMT, social recognition memory test.

bedding were not controlled for. Protocols for care and treatment of animals were approved by the University of California Riverside Institutional Animal Care and Use Committee (AUP#5, 20170026 and 20200018).

2.2 | Experimental design and DE-71 exposure

DE-71 (technical penta-bromodiphenyl oxide; Lot no. 1550OI18A) was obtained from Great Lakes Chemical Corporation (West Lafayette, IN). Two concentrations of DE-71 dosing solutions were prepared in corn oil vehicle (VEH/CON) and used at 2 μL per kg body weight to deliver 0.1 mg/kg/day (L-DE-71) and 0.4 mg/kg/day (H-DE-71). These doses were selected to match the molar concentrations of BDE-47 used in previous mouse studies.[36,61]

Male offspring (F1) were exposed to DE-71 via maternal transfer during a period of 10 weeks encompassing pre-conception, mating, gestation, and lactation (Figure 1A) as described previously.[62] Virgin females were paired with untreated males using harem-style breeding. F1 offspring were weaned at postnatal day (PND) 21 and housed in same-sex cages (2–4/cage) and then subjected to behavioral testing.

Mice were finally sacrificed by exsanguination followed by cervical dislocation under terminal isoflurane anesthesia (5%).

To reduce cross-over effects, behavioral tests were distributed across three different cohorts. Mice in Cohort 1 (at mean age) were evaluated on behavior tests used to determine sensorimotor integration on Suok (PND 43), short-term social recognition memory on social novelty preference test (SNP) (PND 71), sociability on a three-chamber sociability test (SOC) (PND 87), anxiety on elevated plus maze (EPM) (PND 72), and spontaneous locomotor activity on open field test (OFT) (PND 100). Brains harvested from Cohort 1 mice were collected at sacrifice on PND 110 and analyzed via RT-qPCR. Cohort 2 mice were evaluated for repetitive activity on a marble burying (MB) test (PND 81), social odor discrimination on an olfactory habituation/dishabituation test (OHT) (PND 79), non-social odor preference on an olfactory preference test (OFT) (PND 102), and despair-like behavior on a forced swim test (FST) (PND 74). Cohort 3 mice were subjected to several tests starting as juveniles: long-term social recognition memory test (SRMT) (PND 30), juvenile OFT (PND 31), juvenile MB (PND 35), and novel object recognition test (NORT) (PND 111). Brains collected from Cohort 1 mice (PND 110) and a subset of Cohort 3 mice (PND 30) were analyzed for PBDE penetration using mass spectrometry. Plasma from Cohorts 1–3 mice were analyzed for AVP and OXT using enzyme-linked immunosorbent assays (ELISA). Mean values were calculated using a minimum of three replicates from separate experiments. Whenever possible, results were analyzed using the litter as the unit of statistical analysis (values from all individuals/litter were averaged) in order to address litter effects. This included all behavioral results except those obtained from OHT and the neuromolecular analyses.

2.3 | PBDE congener analysis: High-resolution gas chromatography–high-resolution mass spectrometry

PBDE levels were measured in PND 30 whole brain homogenates using high-resolution gas chromatography–high-resolution mass spectrometry (HRGC/HRMS) as described by Schramm et al.[63] PBDE analytes included 37 PBDE congeners (BDE-7, 10, 15, 17, 28, 30, 47, 49, 66, 71, 77, 85, 99, 100, 119, 126, 138, 139, 140, 153, 154, 156, 176, 180, 183, 184, 191, 196, 197, 201, 203, 204, 205, 206, 207, 208, 209). Samples were ground and homogenized to a fine powder under liquid nitrogen. Homogenization of samples (100–200 mg) under liquid nitrogen was followed by addition of CHEM TUBE-Hydromatrix (Agilent Technologies). Samples were then spiked with ^{13}C-labelled PBDE standard mix (BFR-LCS, Wellington Laboratories). For pressurized liquid extraction (Dionex ASE 200), n-hexane/acetone (3:1, v/v) was used at 120°C and 12 MPa. Extracts were vacuum evaporated to reduce sample volume to ~5 mL. Samples were purified and fractionated over an acidic silica, alumina and carbon column. Concentrated extracts were spiked with the recovery standard (BFR-SCS, Wellington Laboratories) yielding an average recovery for ^{13}C-labelled PBDE standards of 40%–120%. Subsequently, samples were analyzed by HRGC/HRMS (Agilent 6890/Thermo MAT95 XL) using electron impact ionization (EI) (specifications are listed in Table S1, Supporting Information). All samples were blank-corrected on a congener-specific basis using the average of three procedural blank samples. Analytes with concentrations lower than three times the standard deviation of the blank values or that were not detected were considered as not detectable (n.d.). The limit of quantification (LOQ) was considered as a signal/noise ratio of 9:1 (Table S2). Congener concentrations that were below the detection limit were assigned a randomly generated value of LOQ/2. Interlaboratory comparisons were considered in confirming the accuracy of our method.

2.4 | PBDE congener analysis: Gas chromatography in tandem with electron capture negative ion mass spectrometry

Concentrations of 26 different PBDE congeners ranging from BDE-30 to BDE-209 were measured in adult (PND110) whole brain homogenates using gas chromatography coupled with electron capture negative ion (ECNI) mass spectrometry (GC/ECNI-MS; Agilent 5975N MS) as described previously.[62] Briefly, brain tissue (0.2–0.5 g) was ground with Na_2SO_4, spiked with two isotopically labeled standards (F-BDE-69 and ^{13}C BDE-209), and then extracted using 50:50 DCM:hexane. Extracts were concentrated, measured for lipid content (gravimetric method), and then purified using acidified silica. Recovery of F-BDE-69 and ^{13}C BDE-209 averaged 91 (±6.9%) and 106 (±19.9%), respectively. All samples were blank-corrected on a congener-specific basis. Method detection limits (MDLs) were estimated using either a signal: noise of 10 or by calculating three times the standard deviation of the value in blank. MDLs differed by congener and ranged from 0.8 (BDE-47) to 6.6 ng/g (BDE-206).

2.5 | Neurobehavioral testing conditions

Mice were acclimated to the testing environment at least 30 min prior to testing. Mouse behavior was scored using automated video-tracking software (Ethovision XT 15, Noldus) or manual scoring software (BORIS[64] or JWatcher), performed blind to treatment by trained observers. Mice were tested during the light phase under normal to bright light conditions. We used low light conditions for some behavior tests (SRMT, SNP, SOC), dark conditions for Suok and normal to bright light for OFT in order to promote decisive exploration in conflict with anxiety. Animals were excluded from the study if they failed to engage in testing (e.g., spent <5 s exploring each object on NOR or <30 s exploring each mouse stimulus on SNP), displayed freezing, jumping, aggressive or excessive grooming behavior, moved a total distance <1000 cm (SNP, SOC) or displayed a side preference on three-chamber apparatus (SOC).

2.6 | Social novelty preference

Social novelty preference (SNP) was conducted and analyzed according to methods adopted from published protocols.[65] After a 30-min

acclimation period in a polycarbonate cage identical to their home cage, the test mouse was exposed to two wire interaction corrals (11 cm height × 10 cm diameter) placed on each side of the cage for 30 additional min. In the first 5-min trial (training trial), the test mouse was allowed to interact with an age- and sex-matched stimulus mouse placed into one corral while the empty corral was removed. After a 30-min retention period in the home cage, in the test trial, the test mouse was exposed to the same stimulus mouse (now familiar mouse) and a novel stimulus mouse each placed in a separate corral for 5 min. Stimulus mice were single-housed in order to preserve their unique scent. Both trials were video-recorded and analyzed using BORIS. Investigation by test mouse was measured as time it spent sniffing each stimulus mouse (snout within 2 cm). Mice were excluded from study if they spent less than 25 s actively investigating the stimulus, which is necessary for memory formation.[66] Test robustness was measured using an Investigation Index (IN) calculated as the ratio of time spent investigating the novel mouse to total investigation time during the training trial. IN = (Train Novel)/(Test Novel + Familiar) (Figure S2). Social recognition is represented as increased investigative behavior toward a novel conspecific versus time spent investigating the familiar conspecific stimulus. To evaluate group differences, a Discrimination Index (DI) was calculated as the ratio of time (T) spent investigating Novel − Familiar/total investigation time in test period.

2.7 | Social recognition memory test

A two-trial SRMT was performed on juvenile test mice to assess long-term social recognition memory.[66,67] Test mice (PND 28–40) were exposed to a younger sex-matched conspecific stimulus mouse (PND 15–32) during two 3-min trials following an intertrial delay of 24 h. After a 1-h acclimation, the test mouse was placed into a test cage (27 × 16 × 12 cm) under dim conditions and allowed to interact with the stimulus mouse for 3 min (Trial 1). In Trial 2, performed 24 h later, the same test mouse was exposed to the familiar stimulus (from Trial 1) or a novel stimulus. Digital recordings were made of test trials, and investigation time was scored by a blind trained observer using BORIS. Other direct-contact behaviors were also noted: grooming, licking, and pawing with the juvenile while inspecting any part of the body surface, as well as sniffing of the mouth, ears, tail, anogenital area, and time in proximity (within 1 cm) of the juvenile.[68] Mice included in final results (~85% of the original population) spent at least 25s actively investigating the stimulus in order to ensure memory formation.[66] To evaluate the differences in ability to form a long-term social memory, a Recognition Index (RI) was calculated as the ratio of the duration of investigations on Day 2 and Day 1 (RI = Time Day2/Time Day1).

2.8 | Three-chamber sociability

A sociability test was performed in a three-chamber apparatus using methods described previously.[69] The day of the experiment the test mouse was allowed to acclimate to the apparatus for 20 min before testing, including 10 min in the central chamber with the doors closed, followed by 10 min in the entire empty arena with the doors open. The subject was then briefly confined to the center chamber while empty inverted wire corrals (non-social stimulus) were placed in the side chambers. A male stimulus mouse, used as the social stimulus, was placed inside one of the corrals. After both stimuli were positioned, the testing began when the doors were reopened, and the mouse was allowed to access all three chambers for 10 min. The number of entries and time spent in the chambers as well as sniffing time while close to stimuli were manually determined from recorded videos using the event-recorder software JWatcher. Sniffing time, measured as the time the test mouse's snout was within 2 cm of the stimulus mouse, has been suggested to have superior validity over chamber time scores since active behaviors that are most directly related to social investigation are captured[70] and the physical proximity allows for transmission of volatile and nonvolatile odorants.[70–72]

2.9 | Marble burying and Nestlet shredding

The MB and the Nestlet test measure repetitive and perseverative behavior preferentially over novelty-induced anxiety in rodents.[73,74] The test mouse was placed in a polycarbonate cage (19 × 29 × 13 cm) with 5 cm deep bedding containing an equidistantly aligned marble array (8 × 4 used for adults or 5 × 4 used for juveniles) and allowed to interact with marbles for 30 min as described.[75] A photograph of the cage was taken prior to and after the test, and images were scored by 2–3 investigators who were blind to treatment. Marbles were considered buried if a minimum of ⅔ of the marble was buried in the 32-marble array (adults) and ½ buried in the 20-marble array (juveniles). The number of buried marbles was then averaged between the scorers (Figure S3). The same mice were subjected to a Nestlet shredding test after a 5-min rest period by placing the test mouse into another similarly configured cage containing a pre-weighted square of cotton fiber Nestlet. After a 30-min period, the remaining Nestlet mass was weighed and the percent Nestlet shredded was calculated.

2.10 | Novel object recognition test

The NORT was used to assess hippocampal-dependent *non-social* recognition memory. A two-day protocol, used to evaluate short- and long-term retention time, was employed. On Day 1, the test mouse was acclimated in a testing open field arena (39 × 39 × 38 cm) for 15 min followed by a 20 min acquisition phase in its home cage as described.[76] During training, the test mouse was placed in the open field containing two identical objects (F vs. F) and allowed to freely explore the environment and objects. After a 30 min retention phase in its home cage the test mouse was returned to the apparatus and allowed to explore a familiar and novel object (F vs. N) during test trial 1. Long-term memory was assessed on Day 2, during test trial 2, after a 24 h retention time, by placing the test mice into the arena containing the familiar and a new novel object (F vs. N′). All train/test trials

lasted 5 min and were video recorded. Preference for the novel object was measured as the ratio of time exploring the novel stimulus relative to the total exploration time. A Discrimination Index (DI) was calculated, to represent NOR memory, as the difference in exploration time between novel and familiar objects relative to total exploration time, where 0 indicates equal preference (DI = (Time Novel − Time Familiar)/(T Novel + Time Familiar)). Objects used were first validated for lack of intrinsic preference and for distinguishing capacity by WT mice. Data was generated using Ethovision video tracking software.

2.11 | Olfactory preference test

To test the ability to detect diverse odorants, test mice were exposed to attractive and aversive odorants on the innate Olfactory Preference Test (OPT) described previously.[77,78] Mice were first acclimated to a large empty test cage (19 × 29 × 13 cm) and after 15 min, were sequentially transferred to one of three other cages every 15 min, each containing a filter paper (2 × 2 cm) treated with 500 μL of a test odorant: 10% peanut butter, 1% vanilla, 1% butyric acid, or deionized water. The four test odorants were presented to the test mouse in a randomized order. Time spent sniffing the filter paper during the 3 min odorant trials was video-recorded and later measured using BORIS video tracking software.

2.12 | Olfactory habituation test

The OHT was used to assess a mouse's ability to detect and differentiate social and non-social odorants.[74,79] Mice were acclimated for 45 min to an empty cage with a cotton-tipped applicator inserted through the water bottle hole. Non-social odors included deionized water, almond, and banana (1:100 solutions prepared from extracts). Two different social odors were obtained on the morning of test day by swiping the applicator across the bottom of one of two cages housing unrelated sex-matched conspecifics which had no previous contact with the test subject. Cages housing 3–4 mice with bedding that was at least 3 days old were used. Stimuli were presented in triplicate in the following order: water, almond, banana, social odor 1, social odor 2. Time spent sniffing the test applicator during each 2 min trial period was digitally recorded and later scored using BORIS video tracking software. Metrics obtained from this test included the decrement in olfactory investigation of the same odor after repeated presentations (habituation) and the duration of response upon presentation of a new odorant (dishabituation).

2.13 | Elevated plus maze

Anxiety was assessed using the EPM (20 × 22 cm; elevated 92 cm), constructed from black Plexiglass as described (Lister, 1987). Two open arms and two closed arms received differential lighting, that is, 300 and 30 LUX, respectively, to create anxiogenic (open) and anxiolytic conditions (closed). At the beginning of each trial, the test mouse was placed in the middle of the apparatus facing the open arm and was allowed to explore the apparatus for a total of 5 min. Activity was monitored by an overhead digital camera and video recordings were analyzed for time spent in each arm and the frequency of entries using BORIS.

2.14 | Forced swim test

The FST was used to assess depressive-like behavior using an apparatus consisting of a vertical Plexiglass cylinder (127 × 305 mm) filled to a depth of 150 mm with water maintained at 23–25°C.[80] Testing was conducted under dim room lighting conditions with an overhead light placed above the cylinders. Mice were placed individually into the water column for a 6 min test trial. After the test, mice were removed and dried before returning to their home cage. Swimming behavior was analyzed during the last 4 min using Ethovision software. Active swimming was considered as time spent struggling/paddling with more than one limb. Drifting or passive movements used to maintain verticality in the water column were considered as time spent immobile.

2.15 | Suok: Sensorimotor integration test

The Suok test uses an elevated platform to analyze anxiety, motor impairments, and motor-vestibular anomalies in mice (Kalueff and Touhimaa[84]). The apparatus consisted of a smooth, thin aluminum rod (2 m long, 3 cm diameter) elevated to 20 cm and fixed to two clear acrylic walls as described.[81] Bilateral to a central segment (38 cm) of the aluminum rod were ten 10 cm segments labeled by line markings visible to the experimenter. Mice were acclimated to a dimly lit testing room for 30–60 min, and several behaviors were scored over a 5 min trial: (1) horizontal and locomotor (normalized) activity assessed by number of segments traveled, (2) sensorimotor coordination measured by the number of hind leg slips and falls from the rod, (3) exploratory behavior such as side looks and head dips, (4) anxiogenic behaviors such as increased latency to leave the central zone and unprotected stretch-attend postures,[82,83] (5) vegetative responses such as urinations and defecation boli combined, and (6) autogrooming behaviors. Hyperactivity, loss of sensorimotor coordination, increased anxiety, and displacement behavior were represented by elevated values for #1, 2, 4, and #5, 6.[83,84] Behaviors were recorded manually by 2–4 experimenters.

2.16 | Open field test

The OFT allows rapid assessment of rodent locomotion, anxiety, and habituation without a training requirement.[85] The testing arena consists of a large, brightly lit top-open Plexiglass square

(39 × 39 × 37.8 cm) designed as an aversive environment for rodents. Mouse activity over a 1-h period was digitally recorded and scored using Ethovision. Measures such as distance traveled, velocity, and total time in periphery (10 cm adjacent to wall) and center of the arena were measured. Time in the periphery represents non-anxious motor activity that has been positively correlated with frequency of entries into open arms on EPM and is negatively correlated to defecation.[86] The latter represented anxious motor activity.[87] Habituation to novelty, a measure of simple learning, was indicated by reduced distance traveled over the 1 h period.[88]

2.17 | RNA extraction from brain micropunches

Around PND 110, mice were sacrificed by exsanguination via cardiac puncture followed by decapitation. Whole brains were rapidly dissected and flash frozen in 2-methylbutane over dry ice and stored at −80°C. Brain cryosections of 300–500 µm were generated and mounted on dry ice-cold sterile glass slides and stored at −80°C. Using the Palkovits micropunch technique, we harvested five regions of interest by employing a handmade microdissecting needle (16-gauge).[89] Tissue punches were immediately homogenized in TRIzol Reagent (Thermo Fisher Scientific, USA) by a hand-held homogenizer and snap frozen over dry ice and stored at −80°C until further use. Sample homogenates were subjected to total RNA isolation using the RNeasy Micro Kit (Qiagen, USA). Purity and quality of RNA were assessed photometrically using the ratio of optical density (OD) at 280 nm versus 260 nm (NanoDrop ND-2000). Samples were excluded from the study if RNA purity (260/230) was <1.4. RNA integrity was assessed using an Agilent 2100 Bioanalyzer.

2.18 | Quantitative polymerase chain reaction

All molecular work was carried out in adherence to MIQE guidelines[90] and previously described.[37] Oligonucleotide PCR primers were designed and then ordered as custom primers or predesigned assays from Integrated DNA Technologies (IDT). Oligonucleotide primers were tested against complementary DNA using RT-PCR and gel electrophoresis. Only primers that gave single-band amplicons in the presence of reverse transcriptase (RT) and that matched the base length of the predicted target and that yielded 90%–110% efficiency were chosen for further use in qPCR. Predesigned assays were validated by IDT. *Oxtr* and the reference gene, *ActB*, were multiplexed using hydrolysis probes with double-quenchers. For all other primers, intercalating dye chemistry was used. Primer sequences have been previously reported.[37] RNA samples (1–4 ng) were processed in triplicate on a CFX Connect thermocycler (Biorad, Hercules, CA) using Luna Universal or Probe one-step qPCR Master Mix (New England Biolabs, Ipswich, MA). No-template controls (NTCs) and negative RT controls were included in each experiment to rule out extraneous nucleic acid contamination and primer dimer formation and the presence of genomic DNA (gDNA), respectively. Gene expression was measured relative to the reference gene, *ActB*, and differential gene expression was determined as fold-change compared to VEH/CON using the Pfaffl method.[91]

2.19 | Enzyme immunoassays

Mice were subjected to CO_2 inhalation and blood was collected by cardiac puncture and the plasma separated at 2000*g* centrifugation for 20 min at 4°C. Plasma levels of the OXT and arginine8-vasopressin (Arg^8-vasopressin) were quantified spectrophotometrically using ELISA kits from Arbor Assays (Ann Arbor, MI; OXT, K048-H1, Arg^8, K049-C1), Enzo Life Sciences (Farmingdale, NY; OXY: ADI901153A0001, Arg^8: ADI-900-017) and Invitrogen (Waltham, MA; OXT, EEL139). For the Arbor Assay kits, samples were first treated using the acetone-based extraction solution followed by vacuum lyophilization of the supernatant. For OXT ELISA (sensitivity 1.7 pg/mL, range 16.38–10,000 pg/mL), the colorimetric reaction product was read as optical density at 450 nm on a plate reader (SpectraMax 190, Molecular Devices). Arg^8-Vasopressin (sensitivity 0.9 pg/mL, range 1.638–1000 pg/mL) was detected using a luminescence plate reader (Victor3, Perkin Elmer). For the Enzo Life Sciences kit, samples underwent solid phase extraction using 200 mg C18 Sep-pak columns as described.[92] Measurements generated with the Invitrogen ELISA had a sensitivity of 9.38 pg/mL in a dynamic range of 15.63–1000 pg/mL. Neuropeptide concentrations in samples were quantified by interpolating absorbance or luminosity values using a 4-parameter-logarithmic standard curve (MyAssays).

2.20 | Statistical analyses

Statistical analyses were conducted using GraphPad Prism (version 8.4.3, San Diego, CA). Data normality was assessed with the Shapiro–Wilk test and the homogeneity of variances across groups was evaluated using the *F* test. For parametric data, both one-tailed unpaired or two-tailed paired Student's *t* tests were used to analyze congener concentrations and performance on the SNP, SRMT, and NOR tests. When comparing more than two groups, one-way ANOVA with Tukey's or Dunnett's post hoc tests were employed. If variances differed significantly, Brown–Forsythe ANOVA or Welch's correction was applied instead. The Kruskal–Wallis *H* test with Dunn's post hoc was used for nonparametric group comparisons. To compare values from social and NOR tests against a hypothetical mean, one-sample *t* tests were used. The significance threshold (α) was set at .05; *F* and *p* values are provided in Statistical Results in Supporting Information. The data are expressed as the mean ± SEM or as median and interquartile range on whisker plots. Statistical outliers were excluded if they exceeded two standard deviations from the group mean, while technical outliers were excluded if animals were unable/unwilling to perform behavioral assessments correctly (see above). Interrater reliability for MB and nest scores was determined by Bland–Altman analysis, reporting bias as the mean difference (with zero indicating

perfect agreement) and precision as the 95% limits of agreement (mean bias ±1.96 SD).

3 | RESULTS

3.1 | DE-71 dosing paradigm and maternal parameters

C57Bl/6 dams were exposed to DE-71 and their F1 male offspring were investigated (Figure 1). Using this dosing paradigm, we have previously reported neurodevelopmental delays that could not be attributed to differences in litter size at birth, secondary sex ratio, gestational maternal parameters nor maternal retrieval behavior.[62,93] Therefore, our current results are interpreted without cause assigned to maternal behavior abnormalities. In this study, offspring body weights, measured at PND 44, were not affected by exposure (Figure S1). This is consistent with our previous report showing no differences in male body weight or composition in adulthood.[94]

3.2 | PBDE congener analysis in offspring brain

PBDE congener content was determined using HRGC/HRMS or GC/ECNI-MS in brains from exposed F1 male juveniles soon after weaning (PND 30) or as adults (PND 110), respectively. Thirty-seven BDE congeners representing di- through deca-BDEs were analyzed since commercial DE-71 mixtures contain primarily tetra-, penta-, and hexa-brominated congeners but also traces of tri- and hepta-brominated congeners.[95] Raw values by l.w. and/or w.w. are listed by exposure group in Tables S3–S5. In Figure 1B mean group values in w.w. of sum concentrations of 12 PBDEs detected ($\sum_{12}$PBDEs) indicated penetration of L-DE-71 ($p < .05$) and H-DE-71 ($p < .01$) into PND 30 offspring, relative to VEH/CON, confirming that the dosing regimen led to maternal transfer of PBDEs to offspring brain in a dose-dependent manner ($p < .05$). Mean $\sum_{12}$BDE values were 45.4 and 147 ng/g w.w. for L-DE-71 and H-DE-71, respectively (Figure 1B).

Collectively, 6 of 12 congeners (BDE-47, -99, -100, -139, -153, -154) in L-DE-71 and H-DE-71 accounted for 97% of all PBDEs penetrating the brain at PND 30. These same six congeners comprise 97% of the DE-71 mixture (Figure 1D). The remaining congeners in our samples (BDE-17, -85, -138, -140, -183, and -184) made up 2.0%–2.8%. However, when compared to VEH/CON, mean concentrations for only BDE-153 ($p < .05$) and BDE-139 ($p = .09$) were comparatively different in L-DE-71; other congeners were limited due to the low dose and sample size (Table S3). In contrast, the mean concentrations for all six major BDE congeners were significantly greater in H-DE-71 versus VEH/CON ($p < .05$–$.0001$). Of the six minor congeners, five (i.e., BDE-85, -138, -140, -183, -184) were also significantly greater in H-DE-71 versus VEH/CON ($p < .05$–$.0001$). Notably, BDE-153 was 12-fold enriched and BDE-47 was ~6-fold reduced relative to levels in the DE-71 mixture as reported previously[96] (Figure 1E). Both BDE-153 and -47 are reported to be transferred from blood serum of pregnant women to cord blood serum of their newborns in a general population cohort.[97]

While the overall magnitude decreased ~75-fold from PND 30 to PND 110, that is, 1.2 and 2.1 ng/g w.w. (or 78.6 and 230 ng/g l.w.) for L- and H-DE-71, respectively, the mean group values for ∑PBDEs show similar penetration trends of L-DE-71 ($p < .05$) and H-DE-71 ($p < .01$) versus VEH/CON at PND 110 (Figure 1C and Table S5). At PND 110 only BDE-28/33 ($p < .01$), BDE-47 ($p < .01$), BDE-100 ($p = .06$), and BDE-153 ($p < .01$) were detected in L-DE-71 and BDE-47 ($p < .0001$), BDE-100 ($p < .01$), BDE-153 ($p < .001$) in H-DE-71 (Figure 1F and Table S5). For H-DE-71 mean concentrations were greater for BDE-47, BDE-100, and BDE-153 ($p < .01$–$.0001$). Figure 1D depicts BDE congeners by percent composition in DE-71 mixture.

3.3 | Perinatal exposure to DE-71-induced ASD-like deficits in F1 male

3.3.1 | Social novelty preference

Testing mice on a social novelty preference (SNP) test has been suggested to be ethologically relevant to symptoms observed in autistic individuals.[65] On this test, the VEH/CON offspring, but not the L-DE-71- or H-DE-71-exposed mice, showed a preference for the novel over familiar stimulus (Figure 2A; $p < .01$). In agreement with this, within group comparisons of mean recognition index (RI) scores showed a significant increase from "0" (a hypothetical value indicating no preference) in VEH/CON but not DE-71-exposed males (Figure 2B; $p < .01$, one-sample t test). A one-way ANOVA for across group comparisons revealed reduced scores for L-DE-71 (apparent, $p = .08$) and H-DE-71 ($p < .05$) as compared to VEH/CON. A dose responsivity curve indicated a marked but not statistically significant negative relationship between dose and investigation time of novel mouse, suggesting an *apparent* dose-dependent effect of DE-71 on social recognition memory ($r^2 = .69$; $p = .19$; $R^2 = .184$, $p = .07$; Figure S2C). Investigation index scores comparing investigation on Trial 2 (test) versus Trial 1 (training), a measure of sociability, indicated that all groups were prosocial and that the reduced exploration of a novel stimulus in L-DE-71- and H-DE-71-exposed offspring was not due to a lack of social interest (Figure S2A).

3.3.2 | Sociability

To determine social interest, an independent social cognition domain, we examined mouse behavior on a three-chamber sociability test. All F1 groups showed preference for a novel social stimulus relative to a non-social novel stimulus as measured by sniffing time (Figure 2C; $p < .05$, $p < .01$), and by time in chamber, both indicating normal sociability (Figure 2D; $p < .05$). As a measure of test robustness, there was no indication of side preference during training (Figure S2).

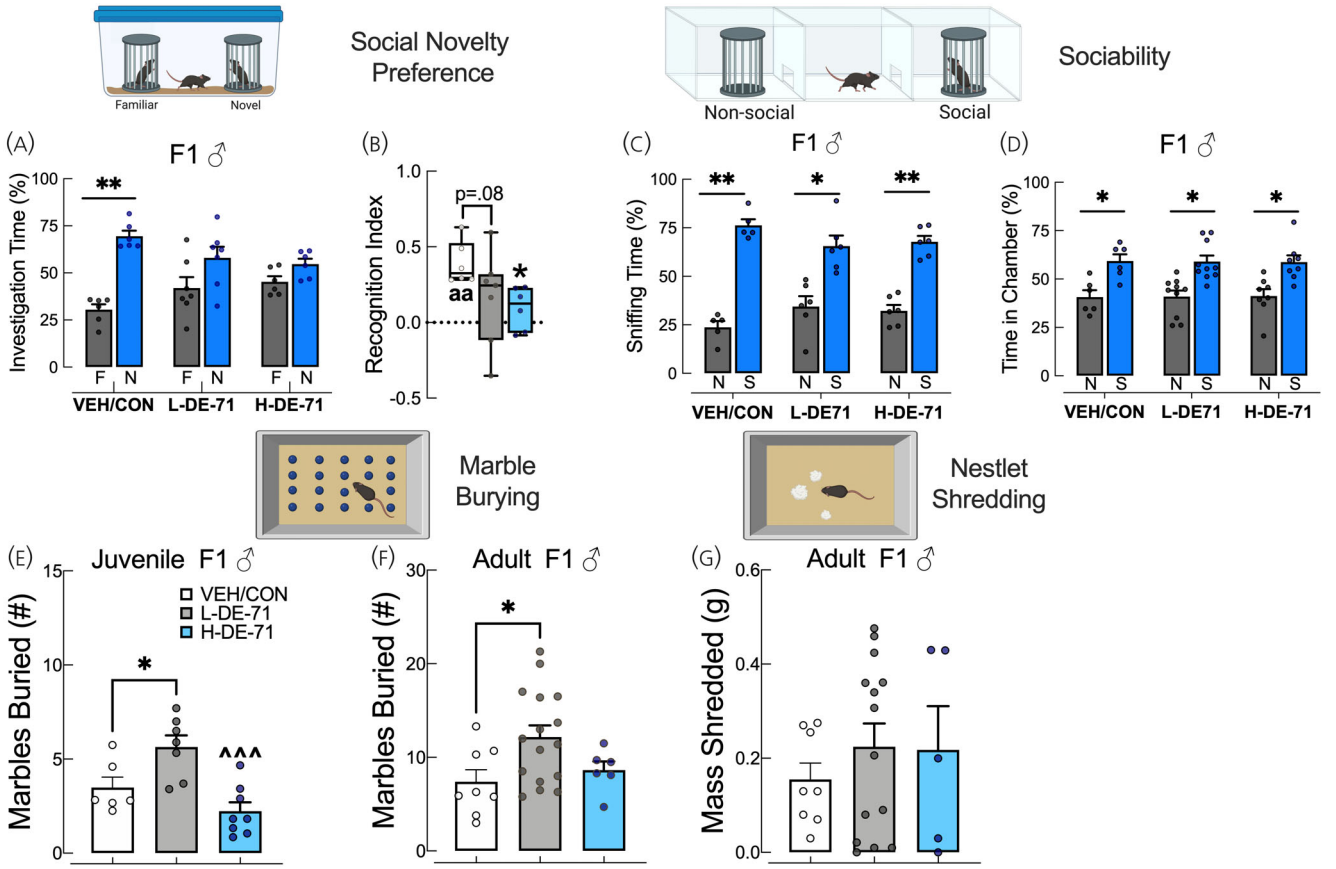

FIGURE 2 Early-life exposure to DE-71 induces deficits relevant to core symptoms of ASD in juvenile and adult F1 males. (A) Social Novelty Preference scores for male offspring: unlike VEH/CON, L-DE-71, and H-DE-71-exposed male offspring failed to prefer a novel (N) relative to a familiar (F) conspecific stimulus. (B) Recognition Index scores showed that only VEH/CON were significantly different from "0" (hypothetical score representing no preference), indicating proper social recognition. (C) Time spent sniffing a social (S) versus non-social (N) stimulus on the Sociability test. All exposure groups spent significantly more time sniffing the social stimulus, indicating normal sociability. (D) Chamber time scores in Sociability test. All groups showed significantly greater time spent with S relative to N. (E) Marble Burying scores in juvenile offspring showed that DE-71 exaggerated digging behavior in a dose-dependent manner. (F) Marble Burying scores in adult offspring showed similar results. (G) Nestlet shredding was not affected in exposed offspring. *Statistical difference from familiar (A) or from non-social stimulus (C, D) or from VEH/CON (B, E, F, G), *$p < .05$, **$p < .01$, [a]Statistical difference versus "0" on a one-sample t test, [aa]$p < .01$. n, 6–7 litters/group (A, B), 5–10 litters/group (C, D), 6–8 litters/group (E), 6–16 litters/group (F), 5–14 litters/group (G). F, familiar; N, novel (A); NS, non-social; S, social.

3.3.3 | Repetitive behavior

On the MB test, L-DE-71, but not H-DE-71, juvenile PND 30 male offspring buried a significantly greater number of marbles relative to VEH/CON (Figure 2E; $p < .05$). Interestingly, H-DE-71 males buried fewer marbles than L-DE-71 males (Figure 2E; $p < .001$). Similar results were found for a subgroup of exposed male offspring tested in adulthood (Figure 2F; $p < .05$). There were no group differences found on the Nestlet shredding test (Figure 2G).

3.4 | Perinatal exposure to L-DE-71 but not H-DE-71 reduced long-term social recognition memory

We determined that SNP scores requiring a 30-min (short-term) memory retention were abnormal in exposed F1 males. To measure group effects on *long-term* social recognition memory, we subjected mice to a SRMT.[66,67] On this test, mice with intact memory exhibit less time investigating a familiar juvenile conspecific 24 h after a first encounter of 3 min.[37] Figure 3A shows that VEH/CON and H-DE-71 mice were able to form a memory for a previously encountered mouse (Day 2 vs. Day 1, $p < .01$ and $p < .05$, respectively). In contrast, L-DE-71 showed no significant difference, representing reduced recognition memory. Recognition Index (RI) analysis was not able to detect a memory deficit since mean values for both DE-71 groups were normal, that is, not significantly different from the hypothetical value of 0.65 (one-sample t test) using calculations from previously published reports on WT mice[67] (Figure 3B). Data from individual mice showed an *apparently* abnormal SRM caused by L-DE-71 (one-sample t test vs. 0.65, $p = .08$; Figure S2D), suggesting a milder effect of L-DE-71 on long-term versus short-term recognition memory. Interestingly, H-DE-71 males displayed *stronger*

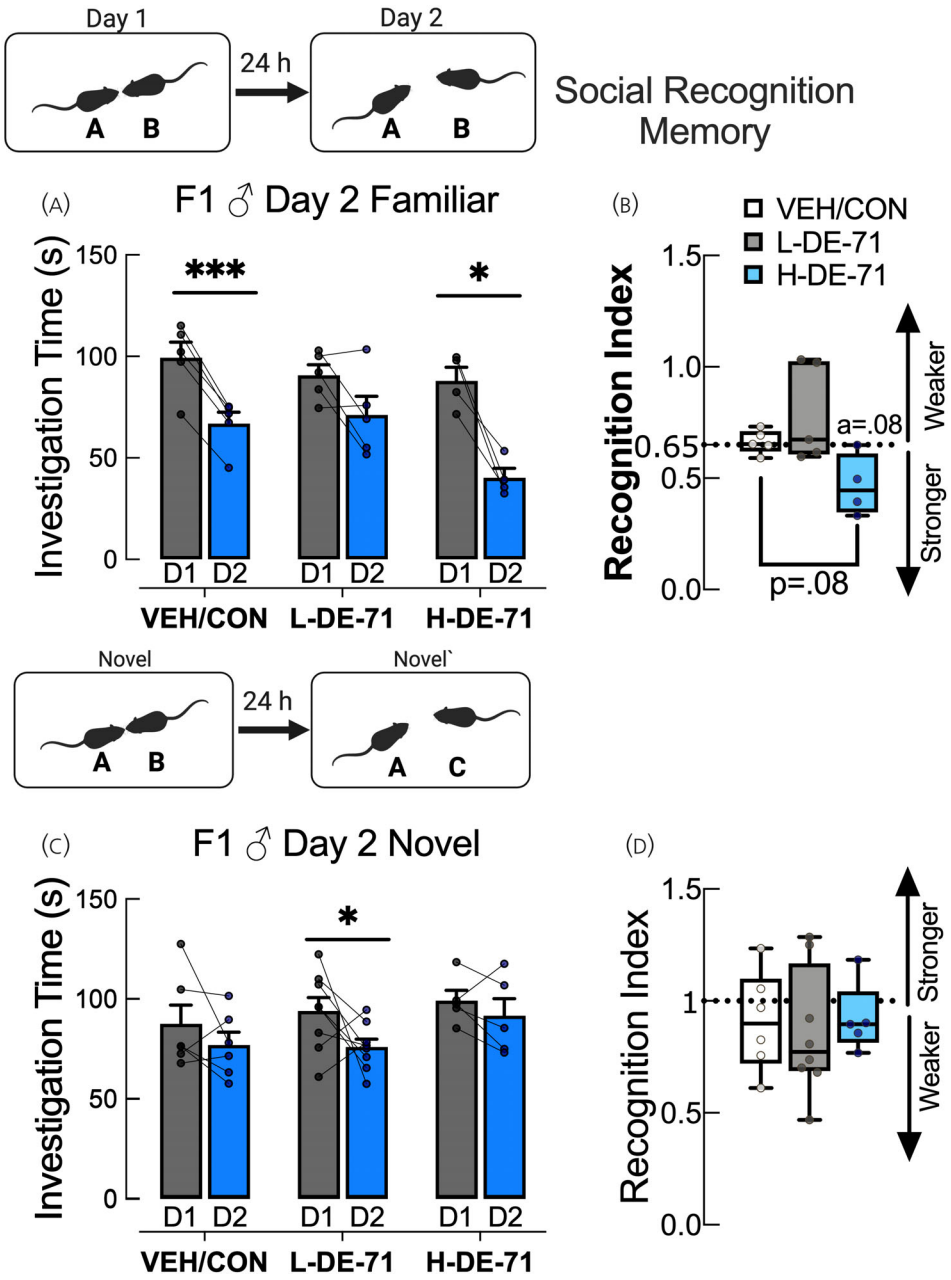

FIGURE 3 Exposure to L-DE-71 compromises long-term social recognition memory (SRM) in adult F1 male mice. (A) When using the same novel stimulus mice on Day 2 (now familiar) the VEH/CON and H-DE-71 test mice (A) displayed a significant reduction in investigation time after the 24 h retention period on Day 2 relative to Day 1, indicating normal SRM. In contrast, L-DE-71 showed no significant difference, representing reduced SRM. (B) Corresponding Recognition Index (RI) scores for familiar were no different from 0.65, the hypothetical value representing normal SRM (one-sample *t* test). (C) When presenting a novel mouse on Day 2 (C, Novel′), VEH/CON and H-DE-71 F1 mice showed equal preference for Novel′, confirming the face validity of the SRM construct. However, L-DE-71 mice displayed reduced investigation of Novel′. (D) Mean RI scores for the novel were not less than the hypothetical value of 1.0, representing normal SRM in this paradigm (one-sample *t* test). *Compared to Day 1, *$p < .05$, ***$p < .001$. *n*, 4–5 litters/group (A); 5–8 litters/group (C). A, test mouse; B, Novel; C, Novel′; D, day.

memory based on their apparently *lower* mean RI score ($p = .08$; Figure 3B). In a separate cohort we examined investigation time with a new novel stimulus mouse on Day 2 to determine whether the reduction of investigation of a familiar mouse on Day 2 is specific to social memory formation or simply disinterest. In Figure 3C, no significant reduction of investigation time was noted as expected for VEH/CON and H-DE-71, suggesting that the reduction in investigating familiar mice in Figure 3A was specific to social recognition memory formation. In contrast, the L-DE-71 group exhibited a significant reduction in investigation time of a different novel mouse on Day 2 ($p < .05$), indicating deficient social memory. Figure 3D shows that RI scores for novel Day 2 memory were not different from the hypothetical value of "1," in which case they would indicate disinterest.[67] In summary, these results indicate that developmental exposure to DE-71 at 0.1 mg/kg/day but not 0.4 mg/kg has mild harmful effects on long-term social recognition memory without effects on social interest.

3.5 | DE-71 compromised short-term novel object recognition memory

Having found that DE-71 exposure produces significant impairment in the SNP and SRM tests, we tested the hypothesis that DE-71 exposure also interferes with non-social recognition memory. Using a novel object recognition memory (NOR) test, Figure 4A shows that L- and H-DE-71 males did not preferentially explore a novel object after a 30-min retention memory of familiar object on

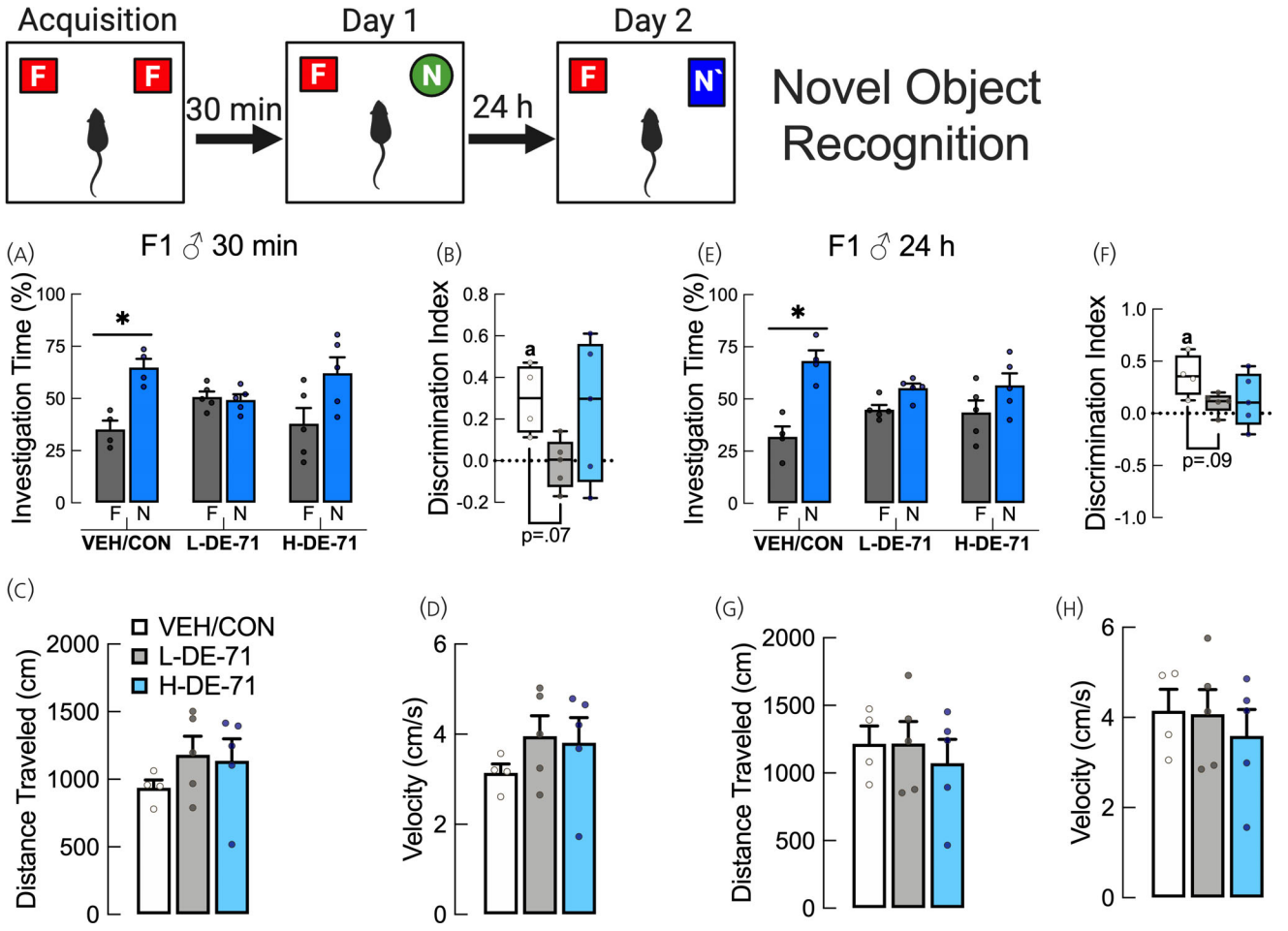

FIGURE 4 Perinatal exposure to L-DE-71 and H-DE-71 compromised short- and long-term novel object recognition memory in adult F1 male mice. (A) Investigation time on short-term novel object recognition test. On the probe trial of Day 1 conducted 30 min after acquisition, exposed male offspring in the VEH/CON, but not L-DE-71 or H-DE-71 groups, showed significantly greater time spent investigating the novel (N) versus familiar (F) objects. (B) Mean discrimination index scores for L- and H-DE-71 males were no different from 0 (no preference) in comparison to that of VEH/CON, which showed preference for the novel object (N). (E) A second probe trial, conducted after a 24-h retention period that measured mean investigation times of a second novel object (N′), indicated deficient object recognition for L- and H-DE-71 groups. (F) Mean discrimination index scores for *long-term* recognition memory were different from the hypothetical value of "0" (no preference) for only VEH/CON. (C, D, G, H) Distance traveled or velocity during the 5 min tests. *Compared to familiar object, *$p < .05$. [a]Compared to hypothetical "0" score, [a]$p < .05$, one-sample t test. n, 4–5 litters/group. (F) familiar object; N, novel object on Day 1; N′, novel object on Day 2.

Day 1 testing, as did the VEH/CON ($p < .05$), indicating that DE-71 alters short-term recognition memory for previously encountered objects. This was corroborated by discrimination index scores (DI) that were greater than 0 (no preference) only for VEH/CON (Figure 4B; $p < .05$, one-sample t test). Additionally, across group comparisons indicated that L-DE-71 had an *apparently* lower DI relative to VEH/CON ($p = .07$). On Day 2, mice were tested for long-term retention of object memory. The VEH/CON ($p < .05$), but neither of DE-71 groups, preferred novel over familiar objects (Figure 4F). This was corroborated by DI scores that were significantly different from 0 for VEH/CON ($p < .01$) but not for L-DE-71 nor H-DE-71. These results suggest that DE-71, at both doses, interfered with short- and long-term object recognition memory. There were no effects of exposure on distance traveled in the testing arena (Figure 4C,D,G,H), excluding the interpretation that locomotor ability confounded the NOR results.

3.6 | Abnormal behavior produced by DE-71 exposure is not due to deficits in general olfactory processing

In order to examine if DE-71-induced deficits observed in SRT, SRMT, NORT were due to insufficient olfactory ability, we subjected male offspring to an olfactory preference test. Figure 5A shows that all mice, including those treated with DE-71, displayed preference for attractive versus aversive odorants, that is they showed increased sniffing duration for peanut butter over water ($p < .01$, $p < .001$), butyric acid ($p < .05$, $p < .01$), and over vanilla ($p < .01$–$.001$).

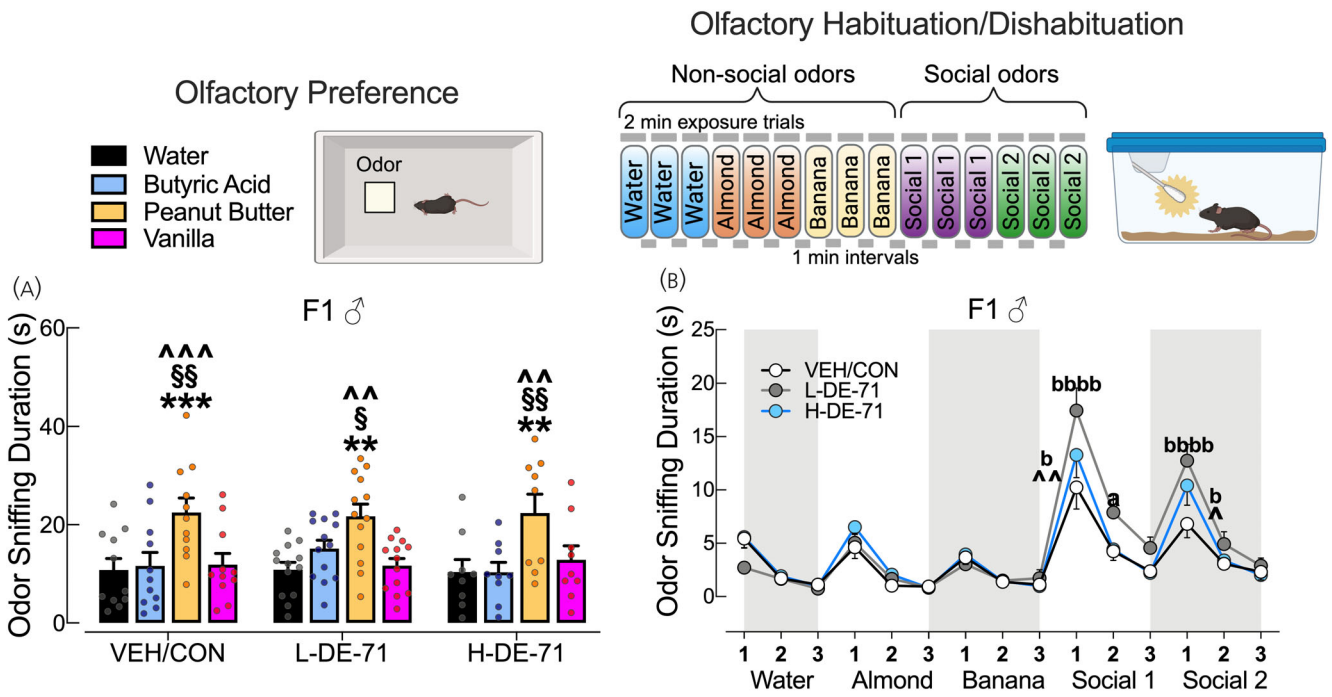

FIGURE 5 Perinatal exposure to DE-71 does not alter general olfaction but disrupts olfactory discrimination of social odors in adult F1 male offspring. (A) DE-71-exposed male offspring showed normal olfactory preference. (B) Sniffing time on olfactory habituation/dishabituation test showed less habituation to social odor 1 in L-DE-71 versus VEH/CON. Both L-DE-71 and H-DE-71 showed abnormally exaggerated dishabituation from banana to social odor 1. *Peanut butter compared to water (W), **$p < .01$, ***$p < .001$; §peanut butter compared to butyric acid (B), $^{\S}p < .05$, $^{\S\S}p < .01$; $^{\wedge}$peanut butter compared to vanilla (V), $^{\wedge\wedge}p < .01$, $^{\wedge\wedge\wedge}p < .001$; ^{a}Statistical difference in habituation to social odor 1 versus VEH/CON, $^{a}p < .05$; ^{b}statistical difference in dishabituation to previous odor versus VEH/CON, $^{b}p < .05$ (H-DE-71), $^{bbbb}p < .0001$ (L-DE-71). Statistical results are summarized in Table 1. *n*, 9–13 litters/group (A), *n*, 16–18 subjects/group (B).

3.7 | DE-71-altered olfactory discrimination of social but not non-social odors

We used an OHT to measure the ability to discriminate non-social from social odors. All groups displayed olfactory habituation to olfactory all non-social odors and social odors (except L-DE-71 did not habituate to non-social odor 2-banana) as indicated by the decline in time spent sniffing odorant at trial 3 (Figure 5B). Table 1 indicates the statistical results of the habituation/dishabituation test. When compared to VEH/CON, both L- and H-DE-71 groups displayed exaggerated dishabituation from non-social 2 to social odor 1 ($p < .0001$ and $p < .05$, respectively) and from social odor 1 to social odor 2 ($p < .0001$ and $p < .05$, respectively). Moreover, L-DE-71 showed deficient habituation to social odor 1 ($p < .05$). These results suggest that DE-71 produces altered olfactory discrimination, especially of social odors, which requires signal processing via medial olfactory epithelium (MOE) and vomeronasal organ (VNO).[98]

3.8 | DE-71 exposure does not promote anxiety nor depressive-like behavior

Male offspring were evaluated for anxiety using the EPM test (Figure S4). Percent time spent in closed arms relative to open arms was significantly greater in all exposure groups ($p < .0001$). There was no group effect of exposure on the number of open arm or total arm entries. Using a FST that measures depressive-like behavior as time spent immobile, we found no significant group effects (Figure S4).

3.9 | DE-71 exposure produced no effects on Suok test

Using Suok, we measured locomotion, exploratory behavior, sensorimotor coordination, and anxiety. There were no group effects detected (Figure S5).

3.10 | Early-life PBDE exposure does not alter locomotion on the OFT

The OFT informs about locomotion, habituation to novelty, and anxiety. H-DE-71 groups showed hyperactivity, relative to VEH/CON, at PND 100, but not at PND 30 (Figure 6A,D). However, this occurred only during the first 5 min of the test (Figure 6D; $p < .05$). These differences were taken into account when interpreting data on NOR and other tests. Time-dependent reduction in exploratory activity

TABLE 1 Statistical results for the olfactory habituation/dishabituation test.

Group	Exposure	Habituation to water	Dishabituation water to non-social odor 1	Habituation to non-social odor 1	Dishabituation non-social odor 1 to non-social odor 2	Habituation to non-social odor 2	Dishabituation non-social odor 2 to social odor 1	Habituation to social odor 1 (Trial 1–2)	Habituation to social odor 1 (Trial 1–3)	Dishabituation social odor 1 to social odor 2	Habituation to social odor 2 (Trial 1–2)	Habituation to social odor 2 (Trial 1–3)
Within Group Comparisons	VEH/CON $n = 16$	$p < .001$	$p < .01$	$p < .01$	$p < .001$	$p < .01$	$p < .001$	$p < .01$	$p < .001$	$p < .001$	$p < .01$	$p < .001$
	L-DE-71 $n = 18$	$p < .0001$	$p < .0001$	$p < .001$	$p < .01$	NS	$p < .0001$	$p < .0001$	$p < .0001$	$p < .0001$	$p < .0001$	$p < .0001$
	H-DE-71 $n = 16$	$p < .001$	$p < .0001$	$p < .0001$	$p < .001$	$p < .01$	$p < .0001$	$p < .001$	$p < .0001$	$p < .001$	$p < .01$	$p < .001$
Between Group Comparisons		NS	NS	NS	NS	NS	VEH/CON vs. L-DE-71, $p < .0001^{bbbb}$ VEH/CON vs. H-DE-71, $p < .05^{b}$ L-DE-71 vs. H-DE-71, $p < .01^{\wedge\wedge}$	VEH/CON vs. L-DE-71, $p < .05^{a}$ L-DE-71 vs. H-DE-71, $p < .05^{\wedge}$	NS	VEH/CON vs. L-DE-71, $p < .0001^{bbbb}$ VEH/CON vs. H-DE-71, $p < .05^{b}$	NS	NS

Note: Data and symbols are shown in Figure 5C,D. Non-social odor 1, almond; non-social odor 2, banana; social odors were obtained from dirty cages of sex-matched unfamiliar conspecifics. Habituation compares to previous trial of same odor. Dishabituation compares to previous odor.

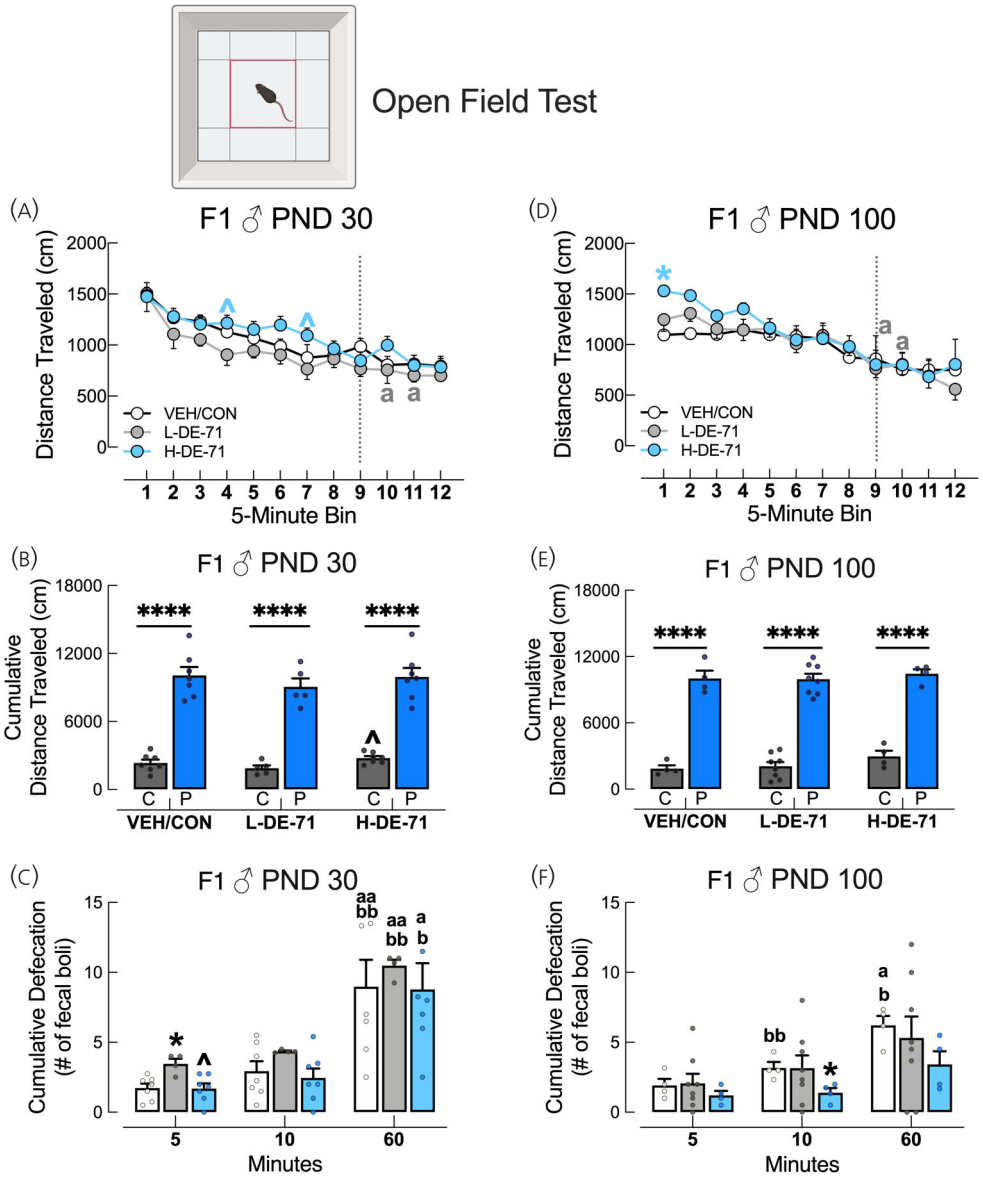

FIGURE 6 Effects of early-life PBDE exposure on open field test performance. (A, D) Distance traveled in the open field arena indicated less habituation in L-DE-71 at PND 30 and early hyperactivity in H-DE-71 at PND 110. (B, E) Exploration time in center was less than in periphery for all groups, suggesting that there are no exposure effects on anxiety. (F) Another measure of anxiety, number of fecal boli, indicated no group differences in emotional reactivity (except L-DE-71 group at 5 min). *Compared to center (B, E) or VEH/CON (C, F), *$p < .05$, ****$p < .0001$. ^Compared to corresponding L-DE-71, ^$p < .05$. For panels A and D: [a]*not* different from initial; time bins lacking an "a" indicate they were significantly different, $p < .01$–$.0001$. For panels C and F: [a]statistical difference versus corresponding group at 10 min, [a]$p < .05$, [aa]$p < .01$; [b]statistical difference versus corresponding group at 5 min, [b]$p < .05$, [bb]$p < .01$. *n*, 4–7 litters/group (A–C); 4–8 litters/group. C, center zone; P, periphery zone.

(habituation) was defined as reduced distance traveled (after 50 min) relative to the start of the test. All groups tested at PND 30 and 100 habituated to the arena with the exception of L-DE-71 males at PND 30 (Figure 6A; $p < .05$–$.001$), indicating reduced learning in this group.

Exploration in the periphery versus center zones was greater for all exposure groups at both ages as expected in the brightly lit conditions (Figure 6B,E; $p < .0001$). Figure 6B shows that total distance traveled in the center was greater for H-DE-71 versus L-DE-71 ($p < .05$). Another measure of anxiety, number of fecal boli deposited on the open field arena, indicated no group differences except PND 30 males exposed to L-DE-71 at 5 min (increased vs. VEH/CON, $p < .05$; Figure 6C) and PND 100 males exposed to H-DE-71 at 10 min (decreased vs. VEH/CON, $p < .05$; Figure 6F). These results indicate minor and transient effects of DE-71 on emotional reactivity while on OFT.

3.11 | DE-71 exposure alters mRNA expression of social neuropeptide markers in social neural network

To correlate the behavioral findings with deregulated gene markers of neuropeptide systems, that are key mediators of complex social behavior, we measured relative mRNA expression for vasopressin (*Avp*), oxytocin (*Oxt*), PACAP (*Adcyap1*) and their receptors. mRNA was obtained from micropunches of discrete brain nuclei involved in social behavior: amygdala (AMG), bed nucleus of the stria terminalis (BNST), lateral septum (LS), PVN and SON and processed using RT-qPCR. Figure 7 shows that *Oxt* mRNA transcripts were increased in L-DE-71 PVN ($p < .01$) and H-DE-71 PVN ($p < .05$). *Avp* was downregulated in L-DE-71 LS ($p < .05$) and *apparently* downregulated in H-DE-71 LS ($p = .07$) but upregulated in L-DE-71 PVN ($p < .01$) and H-DE-71 BNST ($p < .05$). For the PACAP gene, *Adcyap1*, mRNA transcripts were upregulated in H-DE-71 BNST ($p < .05$). With regard to

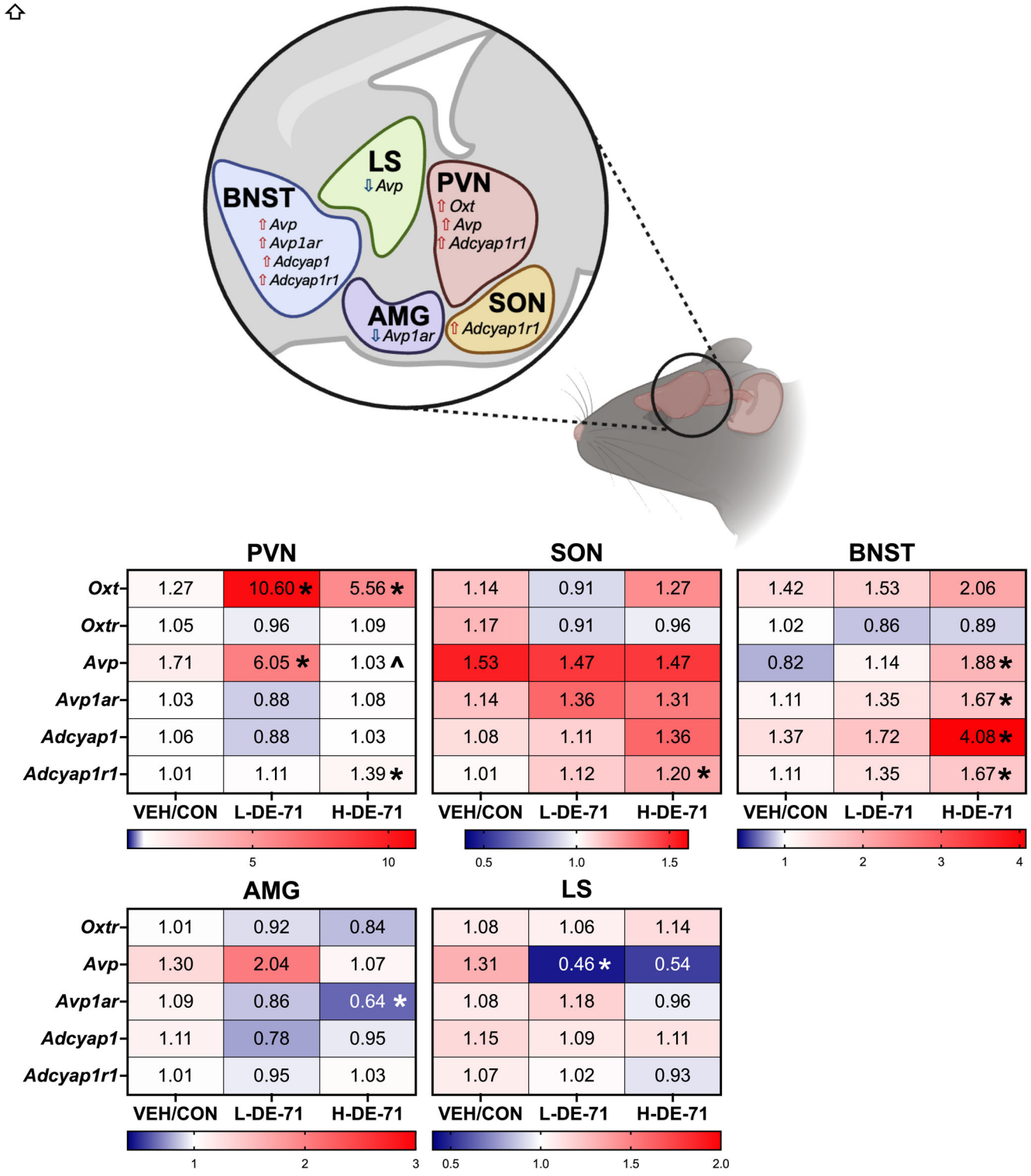

FIGURE 7 DE-71 exposure alters mRNA expression of social neuropeptide markers in select regions of the social neural network in adult F1 male. (top) Anatomical distribution of changes in RNA transcripts. (middle/bottom) Heatmap representation (blue—downregulated; red—upregulated) of RT-qPCR analysis displaying the mean fold-change of each gene in selected SNN brain regions harvested at PND 110. *Compared to VEH/CON, *$p < .05–.01$. ^Compared to L-DE-71, ^$p < .05$. PVN, paraventricular nucleus; SON, supraoptic nucleus; BNST, bed nucleus of the stria terminalis; AMG, amygdala; LS, lateral septum; *n*, 3–14 samples/group.

receptors, *Oxtr* levels were *apparently* decreased in H-DE-71 AMG versus VEH/CON when using a less strict *t* test ($p = .08$) but not when using ANOVA. For *Avp1ar*, levels were upregulated in H-DE-71 BNST ($p < .05$) and downregulated in H-DE-71 AMG ($p < .01$). For the PACAP receptor gene, *Adcyap1r1*, mRNA transcripts were upregulated in H-DE-71 PVN ($p < .01$), SON ($p < .05$) and BNST ($p < .05$).

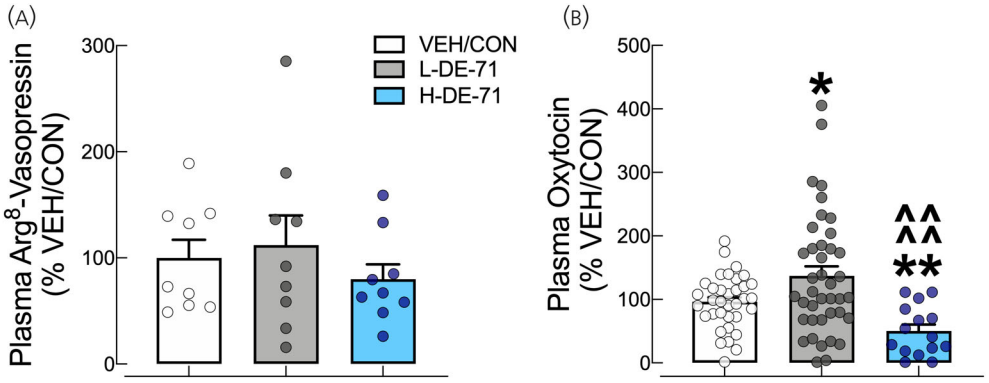

FIGURE 8 Perinatal exposure to DE-71 produced dose-specific responses in plasma OXT levels of adult F1 males. (A) Plasma Arg-8 vasopressin measured using EIA using blood taken from adult male offspring at sacrifice. (B) Oxytocin levels showed dose-dependent increases and decreases in L-DE-71 and H-DE-71, respectively, relative to VEH/CON. *Compared to VEH/CON, *$p < .05$; ^compared to L-DE-71, ^^^^$p < .0001$. *n*, 9 subjects/group (A); *n*, 15–40 subjects/group (B).

3.12 | DE-71 produced dose-dependent changes in adult plasma levels of oxytocin

Next, we measured plasma OXT and Arg8-vasopressin concentrations to examine their association with DE-71-altered social behavior phenotypes. Figure 8A shows that plasma AVP levels in DE-71 males were not different from VEH/CON. In contrast, plasma OXT levels were increased in L-DE-71 ($p < .05$) and decreased in H-DE-71 males ($p < .01$), indicating a dose-specific effect (Figure 8B; $p < .0001$).

4 | DISCUSSION

Mounting evidence links early-life exposure to environmental toxicants with an increased risk of psychosocial neurodevelopmental disorders (NDDs); however, a causal link has not yet been established, and the specific ways in which such exposures intersect with biological pathways are not completely understood. Our study addresses this gap by demonstrating that developmental exposure to the penta-PBDE mixture DE-71 induces long-lasting impairments in ASD-relevant outcomes such as social recognition, repetitive behavior, and social odor discrimination in offspring, providing support for the ASD risk conferred by early-life exposure to PBDEs. These behavioral alterations were accompanied by changes in hypothalamic gene markers of prosocial neuropeptides, OXT and AVP, and their receptors within the social neural network. Moreover, by simulating human perinatal exposure to the larger set of environmentally relevant congeners detected in breast milk and using the litter as the unit of analysis to equalize contribution from separate litters, this study attempts to increase translational validity and rigor to our findings. Importantly, this work extends our previous findings in females to include male offspring, revealing sex-specific effects of PBDE exposure on social behaviors and neuroendocrine pathways. Collectively, our findings underscore the heightened vulnerability of developing social circuits to environmental xenobiotics during the critical postnatal windows of neural development.

The most salient finding of this study was that male offspring perinatally exposed to DE-71 produces abnormal behavioral phenotypes that resemble the two core symptom domains of ASD diagnosis: deficits in social reciprocity and communication and repetitive or stereotyped behaviors.[1] Specifically, DE-71 at two doses, 0.1 and/or 0.4 mg/kg, significantly reduced mean scores on a social novelty preference (SNP) test but did not affect sociability, suggesting selective deficits in social memory but not social interest. In contrast, the effect of DE-71 on juvenile SRM was milder than on adult SNP scores for both doses of DE-71, suggesting that neuroactivity of PBDEs was more pronounced on malleable (short-term) hippocampal-associated processes while still producing milder but detectable effects on more permanent processes required for long-term social recognition memory. Alternatively, but less likely, the strongest PBDE actions may require a delay in manifestation since only SNP testing occurred in adulthood. In humans, the ability to recognize other individuals is essential for the formation of any type of enduring social relationship, providing significance to our findings.[99] Notably, in children with ASD, performance on a facial recognition task has been shown to predict ASD symptom severity.[100] Together, these findings provide evidence that PBDE exposure in early life disrupts normal social recognition memory that may be translatable to human ASD, with effects enduring into adulthood.

Our choice of a 5-min period for familiarization in our SNP test was based on our previous validation that a 3-min exposure (but not 1 min) is sufficient to establish long-term social recognition memory for a familiar conspecific leading to reduced exploration time during a second exposure.[37] We acknowledge that social recognition protocols for exposure differ substantially across laboratories (2–10 min) and that there is no single gold standard for duration needed for familiarization. On rodent sociability and SNP tests, the most significant social approach behavior takes place in the first 5 min of testing.[101] Unlike socially isolated mice, group-housed mice show social memory for a familiar juvenile when tested immediately, 30 min, 24 h, 3 days, and 7 days after only a single 2-min-long interaction.[66] Tanimizu et al. (2017) have demonstrated that 3 but not 1 min of social interaction is

sufficient for consolidation of memory on a long-term SRM test.[67] Therefore, we selected a 5-min familiarization period to balance time needed for social encoding while minimizing stress, particularly in animals with potential neuroendocrine disruption.

DE-71-exposed mice also displayed significantly exaggerated repetitive behavior on a MB test indicative of a second core domain of ASD, that is, restricted and repetitive behaviors. This behavior abnormality is not likely due to an overall locomotor deficit or heightened anxiety, both of which can also signal neurodevelopmental disruption.[73,102] Basal ganglia circuitry is instrumental in the control of repetitive and stereotyped behaviors in both mice and humans[103] and CRISPR-engineered gene deletion, specifically targeting the basal ganglia, can mitigate excessive activity on a MB test in the Fragile X mouse model of ASD.[104] However, it is unlikely that abnormal SNP and SRM was due to a hyperactive phenotype observed as part of MB or during the early part of OFT, since the latter was only evident in H-DE-71 mice at PND110, while SNP deficits were seen in both L- and H-DE-71 adult males. Moreover, deficient SRM was measured in *juvenile* L-DE-71 males. The hyperactivity phenotype has been reported after acute exposure to 0.8 mg/kg BDE-99 at PND 10, suggesting that some congeners included in DE-71 composition may be the neuroactive contributors.[13,105]

As with DE-71-exposed females studied previously,[37] DE-71-exposed male mice showed disruption of another hippocampal-dependent function, that is, object recognition memory.[39,67,106,107] SRM disruption, observed in L- and H-DE-71-exposed males, was coincident with exaggerated olfactory dishabituation when exposed to social odors, without deficiency in general olfactory discrimination. Certain genetic models of ASD, such as BTBR and MALTT inbred mice, also show significantly altered olfactory investigation of novel social odors.[39,67,106,107] Similarly, children with ASD often suffer from altered olfactory function and comorbidities.[2] Other models of abnormal SRM, that lack AVP1AR, do not display deficits in spatial (non-social) memory or general olfactory ability,[40] suggesting that SRM, NOR memory, and olfactory recognition are distinct processes, that they all rely heavily on hippocampal function, and that they differ in their sensitivity to AVP administration. They may share other common neuronal and molecular mechanisms involving OXT.[108–110] Thus, through our comprehensive profiling of the abnormal phenotypes of DE-71-exposed mice, we have shown their convergence with ASD core symptoms and comorbidities, providing an expanded multi-behavioral framework, often missing in other environmental and genetic ASD models.

Human epidemiological studies examining the association between PBDE body burden and social competence and other ASD-related outcomes have yielded mixed findings.[24–26,28,29,111–113] In contrast, experimental ASD models, especially those using endocrine-disrupting mixtures, have provided evidence for harmful effects on social behavior. For example, evidence from kestrels exposed to DE-71 aligns with our results, demonstrating abnormal pair-bonding and courtship behaviors with mates.[114] Similarly, perinatal exposure to the EDC mixtures, NeuroMix, Firemaster 550, disrupts social recognition and partner preference in a sex-dependent manner in developmentally exposed adult rats and voles.[33,34,115] Also, exposure to a weakly estrogenic mixture of PCBs, A1221, has been linked to altered sociosexual preference and social investigation in adult rats.[116,117] In combination with these previous reports, our current findings warn of the reprogramming actions of environmentally relevant mixtures of PBDEs and other EDCs on social brain and general memory circuits, leading to lasting social cognition abnormalities, especially when exposure occurs during critical developmental periods.

Sex-specific differences using our toxicant model of ASD are evident when comparing our present findings using males with previous findings generated using females.[37] Compared to exposed females, exposed males exhibited less pronounced deficits in juvenile long-term social recognition memory. Nevertheless, unlike females which were affected by only L-DE-71, both doses of DE-71 reduced adult SNP in males. Repetitive behaviors were equally exaggerated in L-DE-71 of both sexes while altered outcomes of social odor discrimination were sex-specific, that is, decreased ability in L-DE-71 females and exaggerated in L-DE-71 males. Furthermore, deregulation of hypothalamic pro-social gene markers displayed sex-specific profiles. The mechanisms underlying these effects may be related to sexually dimorphic circuitry/neurochemistry enabling social recognition ability[118–120] and/or differential interaction of PBDEs with reproductive endocrine systems.

Our PCR results reveal sex-specific deregulation of prosocial gene markers, *Oxt*, *Avp*, and their receptors, in key SNN hubs that may provide mechanistic insight into behavioral deficits produced by DE-71 in males and females. Specifically, the OXT/OXTR system was dysregulated less vigorously in exposed males with upregulation observed only in PVN transcript levels of *Oxt* and biphasic changes seen in plasma OXT. In contrast, females manifested downregulation of *Oxt* in SON and BNST and *Oxtr* upregulation in several regions such as PVN, BNST, and AMG.[37] In particular, OXT/OXTR action at the medial amygdala is essential for social recognition in male and female mice and, therefore, *Oxt*/*Oxtr* alterations within these SNN hubs may contribute less to the deficient prosocial behavior in males.[38,119,121,122] In agreement with our findings, Reilly and others (2022) have reported elevated *Oxt* in male PVN after developmental exposure to 0.5, but not 1.0 mg/kg, Aroclor1221, pointing to transcriptional deregulation of the PVN OXTergic system as a common reprogrammable hypothalamic target for EDC actions in males.[55] In humans, manipulation of OXT signaling has been attempted in clinical trials to ameliorate autistic symptoms but with inconsistent results.[123]

Sex-specific gene deregulation by L-DE-71 was also noted for the vasopressinergic system, with *Avp* downregulation observed in the female BNST (L-DE-71) and SON (H-DE-71). In comparison, exposed males also showed upregulated *Avp* in the male BNST and PVN (H-DE-71) and downregulated *Avp* in the LS (L-DE-71), an SNN area with the highest density of sexually dimorphic AVP innervation in the brain. *Avp1ar* was upregulated in male BNST and downregulated in male AMG (H-DE-71). In contrast, DE-71-exposed females showed upregulation of *Avp1ar* in BNST (L-DE-71) and downregulation in SON (H-DE-71). Therefore, while exposed females showed

AVP-related gene deregulation in BNST and SON, exposed males manifested altered *Avp* and *Avp1ar* transcripts in four important regions of the social neural network: BNST, LS, AMG, and PVN, regions that are also critically involved in fear responses and stress coping.

AVP gene deregulation impacts forebrain circuit processes that are critical for the formation and maintenance of social relationships: social recognition, social communication, and aggression.[118] Regulation of social recognition by AVP involves V1a receptors in BNST and LS in both *adult* males and females despite sex differences in AVP fiber and V1aR densities.[120,124,125] However, a recent report by Rigney and others (2024) demonstrated that optogenetic stimulation of BNST AVPergic projections to LS increases social investigation (and anxiety-like behavior) in males but not in females.[120] In our hands, only DE-71-exposed males showed altered *Avp* and/or *Avp1ar* in LS and BNST, potentially making males more susceptible to altered social novelty preference and recognition ability. Interestingly, the male BNST contains a higher V1aR density and resident AVP cells receive more input from the medial amygdala in contrast to females and, accordingly, social stimuli activate BNST AVP cells relatively more in males.[120] The sexually dimorphic role of the BNST in male social recognition and aggression coupled with male-specific downregulation of *Avp1ar* in AMG by DE-71 may converge to produce the deficient social behavior in exposed male offspring reported here. Taken together, social recognition deficits produced by PBDEs may reflect the endocrine disruption of sexually dimorphic neuroendocrine systems, including forebrain OXT and AVP pathways that are critical for social cognition.[126] We speculate that, because the promoter regions of OXT[127] and AVP genes[128] are sensitive to epigenetic modification, developmental DE-71 exposure may allow congeners such as BDE-47 to regulate these genes given their documented global DNA methylation.[36,129]

Early-life exposure to various EDCs (PCBs, Firemaster 500, Bisphenol A) has been demonstrated to modify the hypothalamic OXT and AVP neurochemical systems in a sex-, region-, and treatment-specific manner. EDCs such as Firemaster 550,[130] PCBs including Aroclor 1221 and 1254,[51,55,131] and Bisphenol-A[132] disrupt OXT and/or AVP systems, effects that parallel their impacts on social behavior.[33,34,131,133] Likewise, the sex-dependent effects of DE-71 on *Oxt* and *Avp* may be due, in part, to the estrogenic (BDE-47, 99, and 100), anti-estrogenic, and androgenic activity of some constitutive PBDE congeners, since these genes are regulated by sex-specific reproductive hormones.[60,134,135] Recent human studies have reported a male-selective association of developmental PBDE exposure on poorer social skills and executive function and higher ADHD-related behavioral symptoms in children/adolescents, although the potential entangled role of endocrine disruption has not been defined or addressed.[28,136,137]

DE-71 had a biphasic effect on plasma OXT, with exposure to L- and H-DE-71 leading to significantly elevated and reduced circulating levels, respectively. The non-monotonic dose-response relationship of DE-71 may represent multiple pathways through which toxic PBDEs may signal through high-affinity mechanisms at low doses and engage different low-affinity compensatory biological processes at high doses. Plasma levels of prosocial neuropeptides were measured here since reports show that the peripheral levels may help predict central OXT signaling, although the relationship can be complex.[138] Indeed, central and peripheral OXT release from hypothalamic magnocellular neuroendocrine cell systems can occur concomitantly. Zhang et al. (2021) have shown that selective activation of magnocellular OXT neurons promotes peripheral OXT release and simultaneously facilitates central OXT-mediated social interactions, whereas inhibition of these neurons triggers an opposite effect.[58] Using similar chemogenetic methods, our lab has found that activation of magnocellular OXT neurons leads to both a significant rise in peripheral OXT levels and improved social recognition memory in our DE-71 model of autism (Kozlova & Curras-Collazo, unpublished observations).

Using GC/MS, we confirmed penetration of the major BDE congeners in DE-71 in the brain of PND 30 male juveniles born to exposed dams. The sum concentration (<150 ng/g w.w. or 0.15 ppm) was lower than in our previously reported female brain samples at PND 15 (~250 ng/g w.w.) likely due to sample harvest 15 days after separation from the maternal PBDE source. Using a divisor factor of 0.097 to convert w.w. to l.w. (unpublished observations), we estimate our mean brain ∑PBDE in H- and L-DE-71 males at PND 30 to be similar or 3.2-fold greater, respectively, to serum ∑PBDE in California toddlers, that is, 482 ng/g l.w.,[139] suggesting that our maternal PBDE transfer model is translational to children. As with females, lower but significant ppm levels of ∑PBDEs still remained in adult male brains. However, in adulthood, males showed relatively more remaining congeners (BDE 28, 47, 100, and 153) as compared to adult females, for which only BDE-153 was detected.[37] The congener profile in PND 30 brain samples was dominated by BDE-47, -99, -139, -100, -153, and -154, which accounted for 97% of the mean ∑PBDEs in agreement with DE-71 composition.[96] In general, this congener composition mimics that found in toddler serum and breastmilk and postmortem brain samples, i.e., BDE-47, -99, -100, -153, and -154.[140–143] Importantly, male brains were enriched with BDE-47, the most prevalent PBDE congener in breastmilk which is still occurring in diets sampled worldwide[144] and has been positively associated with autistic features.[24–26,28,29]

5 | CONCLUSIONS

By applying several established behavioral assays to evaluate ASD-relevant phenotypes, we observed that DE-71 exposure specifically impaired social recognition memory, exaggerated repetitive behavior, and disrupted olfactory discrimination of social odors concomitant with deficient novel object recognition memory. Other behavioral domains including anxiety, depression, sensorimotor integration, hyperactivity, or locomotion were largely unaffected, indicating that DE-71's effects are targeted at specific neural circuits rather than general neurological dysfunction. In our environmental model of ASD, we concomitantly observed alterations in gene markers of OXT and AVP systems, prosocial neurotransmitters/neurohormones that, through

their extensive extrahypothalamic projections, play key roles in the social neural network. Disruption in these OXT and AVP systems has been associated with socio-behavioral impairments resembling the core features of autism[145] and replenishment is currently being explored as a promising therapeutic approach for ASD.[42–44] Although past rodent and human studies examining an association between EDCs and ASD risk have yielded mixed results, with the most convincing evidence arising from exposure to EDC mixtures, our findings using the PBDE mixture, DE-71, implicate xenobiotic toxicants as risk factors for neurodevelopmental abnormalities in exposed male and female offspring. Still, only a limited number of studies have directly investigated the effects of PBDE exposure in relation to autism-like phenotypes, highlighting the pressing need for more detailed epidemiological and experimental research on brominated flame retardant POPs and ASD vulnerability, especially since these toxicants are still being generated and prevalent in mother's breastmilk.

Further, our findings indicate that fetal and early postnatal periods represent critical windows of heightened vulnerability to PBDEs and their neurobehavioral reprogramming activity causes enduring ASD-like traits into adulthood. These critical periods of susceptibility in developing organisms are associated with their limited detoxification capacity and the fragile, complex, and precise neurodevelopmental processes governing growth, differentiation, and neural circuit formation.[13]

5.1 | Limitations of the study

Animals in all groups were fed with rodent chow that contained dehulled soybean meal and the corn oil vehicle used in DE-71 solution contained tocopherol, both of which can act as phytoestrogens. Additionally, all mice were housed with corn cob bedding that can contain mycotoxins and other compounds that act as EDCs. Mice were tested during the light phase of their circadian cycle, when they are usually asleep, which may have affected their performance during behavioral testing. Behavioral analyses were mainly analyzed by litter as the unit of statistical analysis and, while this practice reduces intralitter likeness, the relatively lower sample size may be judged to reduce statistical power. Also, due to the number of tests performed, a few tests used individual data.

Mouse models have been argued to provide construct and face validity for human ASD, especially in monogenetic models. Our toxicant model, including its speculated association with disrupted neuropeptide mechanisms of action, will similarly need to be validated with further investigation. Elucidating the transcriptional processes related to gene deregulation of OXT, AVP, and their receptor in the hypothalamic and extra-hypothalamic forebrain regions by reproductive (estrogen)- and thyroid hormone-disrupting properties of PBDEs may prove informational. It should be noted that species differences in PBDE-mediated estrogenic activity may lead to different developmental effects of DE-71 differentiation of sociosexual brain circuits in mice and humans. In rodents, estrogen derived from aromatized testosterone is the dominant masculinizing driver, while direct androgen receptor activation is likely more important in the human brain.

Brain BDE congener levels were quantified with two independent MS methods (GC/ECNI-MS, HRGC/HRMS) due to project limitations but given the internal validation of each we can confirm that the decline measured by PND 110 reflected elimination rather than analytical error. PCR analysis showed sex-specific effects of PBDEs on genes of prosocial neuropeptides and receptors in select social brain regions. However, low cell density in areas such as the amygdala and LS may have reduced RNA yield and increased variability for low-abundance genes. To mitigate this, we optimized primer design and adhered to MIQE guidelines.

Our focus has been on social behavior and the HNS system; however, it is known that exposure to PBDEs can cause a multitude of other biological/health effects including liver and kidney injuries, oxidative stress, dysbiosis, neurotoxicity, genotoxicity, and immune system disorders.[144] Indeed, we have reported that mice exposed to developmental PBDEs and directly exposed dams exhibit physiological features of diabetes and metabolic syndrome, effects that, in the future, could be integrated with the present findings into an adverse outcome pathway for EDC POPs to aid research, clinical, and legislative efforts.[62,94,146]

AUTHOR CONTRIBUTIONS

Gwendolyn M. Gonzalez: Methodology; formal analysis; investigation; visualization; resources; data curation; supervision; funding acquisition; project administration. **Jasmin D. Tran:** Investigation; data curation. **Gregory Lampel:** Methodology; investigation; writing – review and editing; resources; data curation; funding acquisition. **Jack Reid:** Investigation; data curation. **Roberto Gutierrez:** Investigation; writing – review and editing; data curation. **Jordan Tu:** Data curation; formal analysis; investigation; writing – review and editing. **Naran Luvsanravdan:** Formal analysis; investigation; writing – review and editing; data curation; funding acquisition. **Laura M. Anchondo:** Formal analysis; investigation; data curation. **Julia M. Krum:** Methodology; formal analysis; investigation; visualization; resources; data curation; project administration; funding acquisition. **Duraan S. Olomi:** Investigation; data curation. **Luis Campoy:** Formal analysis; investigation; data curation; funding acquisition. **Crystal N. Luna:** Formal analysis; investigation; writing – review and editing; data curation. **Valeria Carrillo:** Investigation; data curation; funding acquisition. **Allison L. Phillips:** Investigation; writing – review and editing; data curation; methodology; formal analysis. **Elena V. Kozlova:** Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; writing – review and editing; software; formal analysis; project administration; data curation; supervision; resources. **Eduardo Monarrez:** Investigation; writing – review and editing; data curation. **Yash Korde:** Investigation; data curation. **Maximillian E. Denys:** Methodology; formal analysis; investigation; resources; data curation; supervision; writing – review and editing; writing – original draft; project administration; funding acquisition. **Maher Blaibel:** Writing – review and editing; investigation. **Kayhon M. Rabbani:** Investigation; writing – review and editing; funding acquisition; data curation. **Gladys Chompre:** Writing – review and editing; data curation; formal analysis. **Simon Kim:** Investigation;

writing – review and editing; data curation. **Anthony E. Bishay:** Formal analysis; investigation; writing – review and editing; visualization; data curation. **Damon Platt:** Investigation; data curation. **Bernhard Henkelmann:** Methodology; validation; formal analysis; investigation; data curation; supervision. **Heather M. Stapleton:** Methodology; validation; formal analysis; investigation; resources; data curation; supervision; project administration; funding acquisition. **Bhuvaneswari D. Chinthirla:** Investigation; data curation. **Karl-Werner Schramm:** Methodology; validation; formal analysis; investigation; resources; data curation; supervision; project administration; funding acquisition. **Margarita C. Curras-Collazo:** Conceptualization; investigation; funding acquisition; writing – review and editing; visualization; validation; methodology; formal analysis; project administration; data curation; resources; supervision; writing – original draft.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Care and treatment of animals was performed in accordance with guidelines from and approved by the University of California, Riverside Institutional Animal Care and Use Committee (AUP# 5, 00170026 and 20200018).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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