Chronic exposure to benzyl butyl phthalate (BBP) alters social interaction and fear conditioning in male adult rats: Alterations in amygdalar MeCP2, ERK1/2 and ERα

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Submitted: 2013-06-26 Accepted: 2013-08-01 Published online: 2013-08-03

Key words: **fear conditioning; MeCP2; phthalates; amygdala; endocrine disruption; hormone; sociability**

Neuroendocrinol Lett 2013; **34**(5):347–358 **PMID:** 23922038 NEL340513A01

Abstract

OBJECTIVES: Benzyl Butyl Phthalate (BBP) is an industrial plasticizer that has an unknown action in the central nervous system. Phthalates have recently been associated with behavioral actions that are linked to their endocrine disrupting properties. The purpose of this study was to investigate the behavioral and molecular effects of BBP treatment in male rats.

DESIGN: Male rats were chronically exposed to BBP in the drinking water (5.0 ppm and 10.0 ppm) throughout adolescence and into the adult phase of life. Their behavior was then assessed in a learning and memory task (fear conditioning), open field exploration and a test of sociability.

RESULTS: BBP treated rats showed decreased freezing in fear conditioning, no changes in open field exploration, and increased aberrant social behavior. Rats were sacrificed at post natal day 140 and blood and brains were harvested and processed. We found increased hormonally active estrogen, 17-β estradiol, in the serum of BBP treated rats. BBP treatment also induced changes in amygdalar proteins related to synaptic plasticity including decreased MeCP2 levels that correlated with tests of sociability with no changes in stress related proteins such as nuclear factor kappa B (NFkB). We also found alterations in physiological responses as measured by body weight without changes in food consumption suggesting disruption of metabolism and body homeostasis.

CONCLUSIONS: We suggest that BBP administration disrupts normal learning and social behavior, and that these effects could be related to alterations of amygdala function.

ORIGINAL ARTICLE

Abbreviations:

ANOVA	- analysis of variance
BBP	- Benzyl Butyl Phthalate
BPA	- Bisphenol A
CS	- Conditioned Stimulus
CR	- Conditioned Response
DBP	- di-n-butyl phthalates
DEHP	- di (2-ethylhexyl) phthalate
DNA	- Deoxyribonucleic acid
EDC	- Endocrine-disruptor chemicals
ERK1/2	- Extracellular Related Kinase isoform 1/2
GAPDH	- Glyceraldehyde-3-phosphate dehydrogenase
GC-MSD	- Gas Chromatograph Mass Spectrometric Detector
kD	- Kilodalton
MAPs	- Mitogen Activated Protein Kinases
MeCP2	- Methyl CpG binding protein 2
NIH	- National Institute of Health
NFkB	- Nuclear factor kappa B
PDMS-DVB	- Polydimethylsiloxane divinylbenzene
PND	- Post Natal Day
PPM	- Parts per million
PVC	- Polyvinyl chloride
SDS	- Sodium Dodecyl Sulfate
SEM	- Standard Error Mean
siRNA	- Small interfering RNA
US	- Unconditioned Stimulus
VEH	- Vehicle

INTRODUCTION

Phthalates, diesters of benzenedicarboxylic acid, which can be classified as endocrine disrupting chemicals (EDC) are widely used as an industrial plasticizer and are ubiquitous chemical products in today's consumer world. Phthalates increase the flexibility and durability of plastic polymers and are mainly found in polyvinyl chloride (PVC) which is widely used in children's toys, tile flooring, medical tubing blood storage bags, imitation leather, food packaging materials and more (Calafat *et al.* 2006; Silva *et al.* 2006; Lassen *et al.* 2009). Phthalates are also commonly found in industrial settings, landfills and waste disposal sites where the runoffs from these manufacturing sites have been known to affect the nearby wild and marine life (Lassen *et al.* 2009). The majority of polycarbonate plastic products contain phthalates and exposure is unavoidable. Phthalates are not covalently bound to polymers such as plastics, and therefore they leach out of products and come into contact with humans. Phthalates that leach can then gain access to the body through consumption, and do not bind to blood plasma proteins causing damage to deoxyribonucleic acid (DNA) in human sperm (Duty *et al.* 2003; Hauser *et al.* 2006; Hauser *et al.* 2007). Many common phthalates and common EDCs accumulate in fatty tissue and have the ability to transfer across the blood-brain barrier (Jang *et al.* 2012). Circulating phthalates can remain unregulated and potent even at low doses and have active metabolites (Calafat *et al.* 2006; Silva *et al.* 2006) that are often understudied (Koch *et al.* 2004). Similar EDCs such as bisphenol A (BPA) have been detected in measurable concentrations in children (Edington & Ritter 2008), laboratory rodent studies (Richter *et al.* 2007), and are a pervasive presence in marine environments (Oehlmann *et al.* 2008). Benzyl butyl phthalate (BBP) is a less extensively studied compound in mammals. Through gene microarray analysis, BBP has been shown to exhibit a similar gene expression profile as the hormone natural estrogen, indicating its potential as an estrogen mimicker with the ability to impact some of the same pathways and genes as natural estrogen (Parveen *et al.* 2008). *In utero*, lactational, or postnatal administration of di (2-ethylhexyl) phthalate (DEHP), chemically similar to BBP, can adversely affect biosynthesis of estrogens. It is likely that exposure to phthalates pre or post natal can produce permanent alterations and involve epigenetic changes to the genome (Panzica *et al.* 2011).

Phthalates and other EDCs such as BBP are widely known to negatively affect reproductive systems in humans and animals. In humans, exposure to EDCs during sexual differentiation can produce malformations of the reproductive tract, produce antiandrogenic activity (Lyche *et al.* 2009; Swan 2008) and affect reproductive outcomes (Swan 2008). In animal toxicology studies, exposure to EDCs can induce malformations in male rats (Gray *et al.* 2000; Mylchreest *et al.* 2000) reduce fertility and alter ovarian function in female rats (Gray *et al.* 2006). There is extensive data indicating that phthalates have the potential to adversely affect reproductive functions, endocrine outcomes and sexually dimorphic behavior, yet there is a paucity of data examining the social and emotional behavioral outcomes after phthalate and specifically BBP exposure. Discrepancies also exist regarding the full neurobiological repercussions of human exposure to the immense range of environmental toxins and there is a need to characterize compounds such as BBP in well-established animal models. Additionally, the consequences of exposure to phthalates in learning and memory processes remain largely unknown. Therefore, the current study was designed to examine alterations in associative learning and social interaction after postnatal exposure of BBP on Sprague Dawley rats. It was hypothesized that exposure to phthalates during adolescence and into adulthood would negatively impact learning and memory and alter social interaction. Because both learning and memory (Schafe *et al.* 2000) and social responding (Adachi *et al.* 2009) have well known molecular correlates, we examined amygdalar methyl-CpG-binding protein 2 (MeCP2) and extracellular related kinase isoform 1/2 (ERK1/2) protein expression. These proteins are critical to normal cognitive functioning and neuronal development.

MATERIALS AND METHODS

Subjects: Male rats with chronic access to BBP treated water

Twenty four male Sprague-Dawley rats, 4–5 weeks old upon arrival, were purchased from Harlan Laboratories (Indianapolis, IN). Rats were housed in pairs and fed

standard laboratory chow. They were maintained in the colony for at least 1 week prior to experimentation on a 12 hour light/dark cycle. BBP water (5.0 parts per million (ppm) and 10.0 ppm) was offered ad libitum as their only source of fluid intake. Pilot studies determined no difference in fluid consumption between treatment and controls (please see below). Dietary consumption of chow intake was determined by subtracting the weight of the amount of food offered from that not ingested. Body weight was monitored several times per week throughout the study. All behavioral testing occurred during the light portion of the cycle. At approximately 20 weeks of life, the rats were sacrificed by rapid decapitation, see Schema 1 for experimental design. The brains were processed for further biochemical analysis of protein markers. All experiments were approved by the Quinnipiac University Institutional Animal Use and Care Committee and were conducted in accordance with the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*.

Drugs

BBP (benzyl butyl phthalate, cat. 308501 Sigma Aldrich, St. Louis, MO) stock solutions prepared by dissolving it in acetone (Sigma Aldrich, St. Louis, MO) and tap water containing 2% saccharin (Sigma Aldrich, St. Louis, MO) and were administered in the drinking water at doses of 5.0 ppm and 10.0 ppm which are equivalent to the concentrations 5 μg/mL and 10 μg/mL respectively. Fresh solutions were prepared every two days. Vehicle (VEH) solutions contained 2% saccharin dissolved in tap water.

Exposure to BPP in drinking water

Duration of exposure

BBP exposure began at the start of the experiment at Post Natal Day (PND) 40 and lasted until the end of the experiment, PND 140. Once the BBP solutions were prepared, animals were presented with BBP in place of their normal drinking water so that BBP dosed water provided the only source of hydration throughout the exposure period. The BBP solutions were placed in 16 ounce (473 mL) clean glass water bottles with standard rubber stoppers and protected from ambient light exposure. The entire chronic oral exposure period was 100 days. Solutions were completely replaced after 48 hours and excess BBP was properly disposed of and considered environmental waste.

Consumption measures

In a separate cohort of 24 male 12 week old Sprague-Dawley rats, pilot data was gathered related to BBP consumption over a 24 hour period. The procedures for BBP preparation were identical to above. To generate the average daily dose of BBP, we weighed the bottles and divided the consumption values by the total body weight of the animals in the cage and accounted for the concentration of BBP.

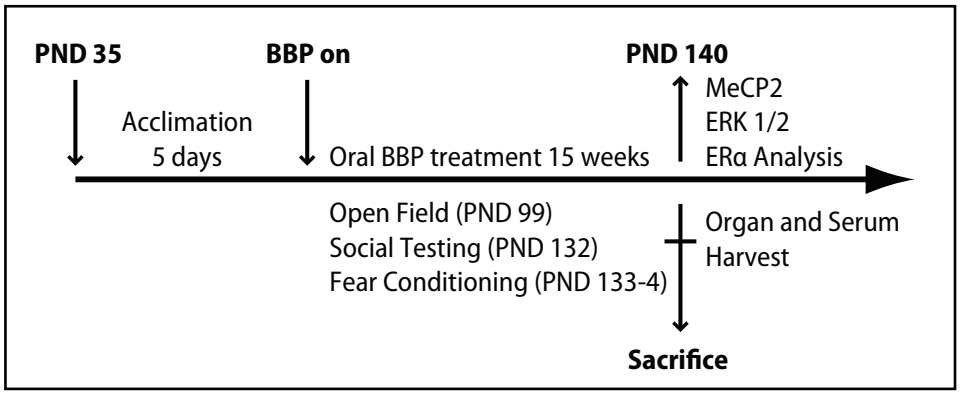
Chemical analysis of BBP

In order to ensure that BBP did not degrade in the drinking water, we used gas chromatography to detect its presence over a series of time points. Chemical analyses of water containing BBP samples were performed using an Agilent Technologies Model 6850 Gas Chromatograph attached to an Agilent 5973N Mass Spectrometric Detector (GC-MSD). An Agilent Technologies HP-5MS, 20 m × 0.25 μm capillary column equipped with a Merlin High Pressure Septum (Supelco Co., 24816-U) was used for analyte separation. Experimental conditions were: initial temperature of 110 °C with a ramp of 5 °C/minutes, to 250 °C, and a total run time of 40 minutes. Extraction of all water samples were performed using a polydimethylsiloxane divinylbenzene fiber (65 μm) with a fused silica core (Supelco Inc., 57346-U) solid phase microextraction assembly (Supelco Inc., 57330U) that was capable of specific adsorption of BBP in the water.

Behavioral tests

Open field testing for gross motor deficits

This behavioral test was performed on PND 99 in a large Plexiglass™ open field arena (113 × 113 × 44 cm). The floor beneath the Plexiglass™ was painted black with white lines marking 23 cm intervals forming a



Schema 1. The experimental design.

5×5 grid pattern on the floor. Rats were placed in a corner of the open field (placement was randomized) and allowed to freely explore for the duration of the test session (5 minutes). All testing was done in a dark room; the chamber was illuminated by a red light. Behavior was recorded by a trained observer, who was unaware of the experimental conditions. For horizontal locomotion, a single activity count was defined as a complete crossing from one black square to another, at the end of which the animal had both hind-paws completely over the white line. Rearing and grooming was recorded simultaneously. Rearing was counted every time the animal stood up with both forepaws in the air or against the wall. Grooming was counted every time the animal cleaned themselves.

Aberrant sociability analysis via anosmia

A 5% zinc sulfate (Sigma Aldrich, St. Louis, MO) solution was administered intranasally with a beveled plastic syringe to gender and age matched naive rats in order to inactivate smell on PND 132. This model renders an animal "passive" by destroying the olfactory epithelium with intranasal lavage of zinc sulfate to produce anosmia. After one hour they were allowed to socialize with BBP treated rats in a sociability chamber (30.5×41.9 cm) for 5 minutes. The social chamber consisted of a box with a removable partition separating the box into two chambers. After a 1 minute habituation period, the partition was removed, both the anosmic and BBP treated rats were allowed to interact and observed for the remainder of 4 minutes. The chamber was wiped with 70% isopropyl between trials. All testing occurred in a quiet sound attenuated cubicle with dim red lights. Scoring consisted of recording sniffing, fighting, mounting, approaching, and grooming in proximity of the anosmic rat and was recorded by an observer who was blind to the experimental treatments. We further defined these behaviors into contact (fighting and mounting), non-contact (approaching and sniffing), and self-directed contact (grooming).

Fear conditioning

In this test, rats learn to associate the context (altered environment) or a cue (auditory tone) with a foot shock. Fear conditioning training (hereafter referred to as Day 1) was conducted on PND 133 in a dark and quiet operant box (Coulbourn Instruments) with standard grid floors wiped with 70% ethanol. The training consisted of a two minute habituation period followed by a three 30-second tones co-terminating with a 2-second, 0.5 mA foot shock pairing. The tones, the conditioned stimulus (CS) was an environmental stimulus that was originally neutral, but acquired salience and eventually predicted the aversive shock, the unconditioned stimulus (US). The inter-trial interval (ITI) between pairings was 2 minutes. Animals were removed from the box 2 minutes after the last foot shock and immediately returned to their home cage. Rats were in the operant box for a total of 10.5 minutes. Twenty four hours following fear conditioning training and on PND 134, animals were placed in the same box but with different context with different floors, ceilings and olfactory cues, for reactivation training (hereafter referred to as Day 2). Reactivation training consisted of 3 CS tones with no concurrent shock, the US. The conditioned response (CR), freezing, was measured for the duration of the session (9.5 minutes) on Day 2. This was done to ensure specificity to the effect of the memory for the CS, not the context, and to shift more towards activation of amygdala-dependent learning. The chamber was wiped with 70% isopropyl between trials.

Molecular analysis

17-β estradiol ELISA

To quantify the effects of chronic oral BBP on blood serum estrogen in the biologically active form of 17-β estradiol, we collected trunk blood during sacrifice at PND 140. Blood was allowed to clot and discarded. Serum was removed and then centrifuged (10,000 revolutions per minute for 10 minutes), extracted and stored at –20 °C until use. Once defrosted, a diethyl ether liquid-liquid extraction was performed and serum was analyzed by ELISA (Enzo Life Sciences; ADI-900-174) following standard protocols.

Western blotting

Bilateral amygdala was dissected from fresh frozen brains in 1 mm punches (ASI Instruments). Tissue punches were homogenized in 1% sodium dodecyl sulfate (SDS) in distilled water with 1:100 phosphatase inhibitor cocktails I and II (Sigma Aldrich, St. Louis, MO). Following homogenization, the samples were assayed for protein concentration using the BCA kit (Pierce Protein Research). Equal amounts of cell lysate protein extracts (15 μg) were subjected to 10–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring to nitrocellulose membrane. The membrane was immunoblotted with different antibodies: ERK1/2 (1:2000 Cell Signaling Technology), p50 (1:1000; Santa Cruz Biotechnology), p65 (1:1000; Santa Cruz Technology) ERα (1:1000; Enzo Life Sciences, ADI-SRA-1010) and MeCP2 (1:1000, Cell Signaling Technology). The housekeeping protein, GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), was used as the endogenous loading control (1:1000; Cell Signaling Technology) for protein normalization. For immunoblot imaging, chemiluminesence (Cell Signaling Technology) was performed following primary and secondary antibody incubation. Relative protein expression was identified using densitometry (Kodak Image Station) and quantified using ImageJ (NIH) or Image Studio Lite (Licor Inc.). The expression of each protein band was normalized to GAPDH for individual samples. For each gel, bands were further normalized to the average of VEH-treated groups to control for between gel variations.

Statistical analysis

Western blots and ELISA

For Western Blots, statistical analysis was done by expressing ratios of protein levels to GAPDH. Data were expressed as the average of the normalized data +/– standard error of the mean (SEM) as compared to controls. For both Western Blot and ELISA, one way ANOVA was used to determine significant differences between groups, with significant differences considered $p<0.05$. Statistical outliers were excluded from analyses, and identified using standard criteria of greater than 2 standard deviations from the mean.

Statistical analysis for behavior

Behavioral and physiological dependent variables were analyzed for group effects by means of Students t tests, one-way, multivariate or repeated measures ANOVA and expressed as treatment group averages +/– the SEM. Fisher's Least Significant Difference test or simple t-tests were used to make post hoc comparisons. Body weight during the maintenance phase (after 14 weeks of age) was analyzed in intervals consisting of 4–7 day bins to smooth daily variations using a repeated measures and multivariate ANOVA. This is a standard analysis in toxicology studies.

RESULTS

BPP in drinking water

Consumption of BPP in drinking water

In the authors' hands, 12 week old adult male rats typically consume on average 80 mL of fluid per day. Over the course of the pilot period, the average BBP doses observed were 2.0 mg/kg/day for the rats consuming a concentration of 5.0 µg/mL (5.0 ppm dose) and 4.0 mg/kg/day for the rats consuming a concentration of 10.0 µg/mL (10.0 ppm dose). We have observed no evidence of a difference in preference for BBP treated water versus non-treated water ($F_{(2,21)}=0.18$; p=n.s.) in a one-way ANOVA analysis for dose x fluid consumed.

Detection of BBP in drinking water

The VEH solutions (1% saccharin and tap water) contained no traceable amounts of BBP. Our high dose, 10.0 ppm, contained approximately twice the absolute amount of our moderate dose, 5.0 ppm (see Table 1) after 48 hours of shelf life. We determined that it was optimal to change BBP solutions after 48 hours to prevent degradation.

Physiological responses to BBP

BBP treated animals did not show fluctuations in body weight during the growth phase ($F_{(2,21)}=1.18$; p=n.s.) by repeated measures ANOVA, see Figure 1A. There was no interaction between dose and time in this phase ($F_{(14,147)}=1.35$; p=n.s.). A repeated measures ANOVA for the maintenance phase showed no significant effect of dose ($F_{(2,20)}=1.56$; p=n.s.) or interaction between dose and time ($F_{(4,40)}=0.61$; p=n.s.). However, given that 5.0 ppm BBP-exposed rats appeared to have not gained as much weight as control rats in the maintenance phase and in light of literature suggesting non-monotonic relationships for low dose endocrine disruptors (Fagin 2012) we performed further analysis. To examine non-linear trends we found a significant

Tab. 1. Demonstration that BBP is detectable in BBP treated water after 48 hours.

Sample (5min)	Trial 1 Avg Peak Area (Hz)	Trial 2 Avg Peak Area (Hz)
VEH	Negligible	Negligible
5.0 ppm	1.45 e^6	1.38 e^6
10.0 ppm	2.42 e^6	2.07 e^6

Samples of BBP spiked water were subjected to gas chromatography attached to a mass spectrometric detector. Samples without BBP showed negligible amounts of BBP whereas the 5.0 ppm and 10.0 ppm detected the presence of BBP using the average peak area in the correct ratio.

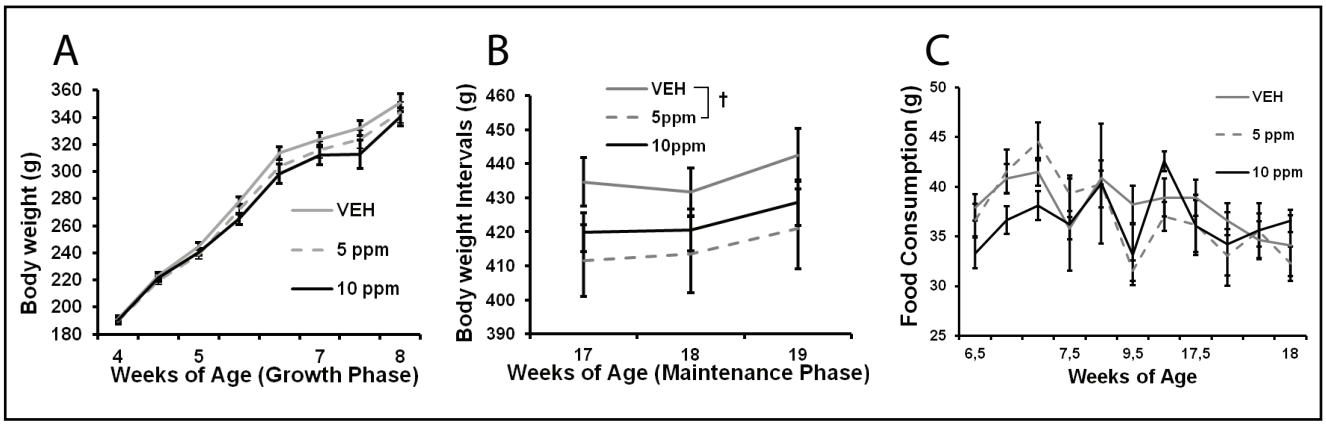


Fig. 1. Physiological responses to BBP exposed rats. Fig 1A and 1B show body weight measures through juvenile and adult phase of rats exposed to BBP at 6.5 weeks until approximately 20 weeks of age. Rats showed no difference in body weight during their growth phase but showed slight differences in the maintenance phase in the 5.0 ppm group. Fig 1C shows food consumption remained the same throughout both phases of growth. Data represent group means ± SEM; †$p<0.09$

quadratic interaction of dose and time ($F_{(2,20)}$=3.31; p=0.057) and a significant interaction by multivariate ANOVA ($F_{(4,38)}$=2.72; p<0.05). Pairwise comparisons showed that the treatment group, 5.0 ppm, bordered on significance with a trend effect for lower body weight (p<0.10). Lastly, student t-tests displayed statistically significant or trend differences between VEH and 5.0 ppm at each body interval (p<0.05; p=0.09; p=0.07), see Figure 1B. Food intake across the entirety of the study was not significantly different in daily variations, ($F_{(2,9)}$=0.55; p=n.s.) by repeated measures ANOVA, see Figure 1C. There was no interaction between dose and time in food consumption ($F_{(22,99)}$=1.48; p=n.s.). There were no statistical differences between BBP treated animals and controls with circulating 17-β estradiol ($F_{(2,21)}$=0.6; p=n.s.) by one-way ANOVA, see Figure 5A.

Behavioral data after BBP administration

Open field activity and the impact of BBP

BBP treated animals did not display any gross motor or exploration deficits. There were no statistical differences by one-way ANOVA in activity in the outer portion ($F_{(2,21)}$=0.26; p=n.s.) or inner portion ($F_{(2,21)}$=0.34; p=n.s.) of the open field arena, see Figure 2A. During this test we also assessed grooming and rearing and there were no statistical differences via one-way ANOVA ($F_{(2,21)}$=0.28; p=n.s.) and ($F_{(2,21)}$=0.87; p=n.s.) respectively, see Figure 2B.

Fear conditioning and the Impact of BBP

We assessed learning and memory in BBP treated rats by using the fear conditioning paradigm. Freezing during training sessions on Day 1 was examined via video and behavior between controls and BBP treated were indis-

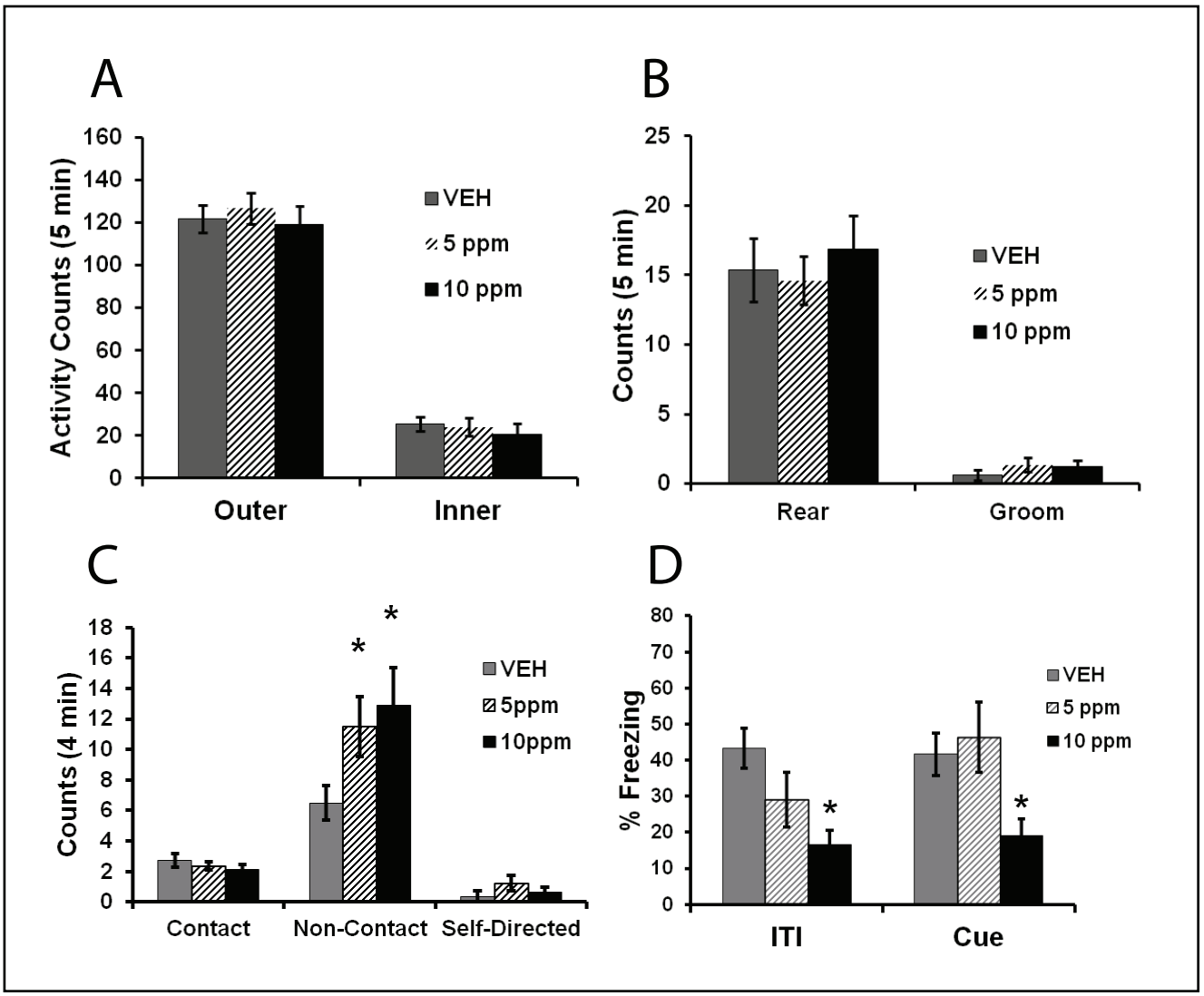


Fig. 2. Behavioral testing in rats chronically exposed to oral BBP. In Fig. 2A-2B, rats exposed to BBP displayed no gross motor deficits, exploration or changes in rearing and grooming in the Open Field test. Fig. 2C shows freezing behavior on Day 2 after rats were returned to the chamber. Rats exposed to 10.0 ppm BBP displayed decreased expression of fear (freezing) during the ITI and Cue phase of fear conditioning. Fig. 2D shows rats treated with chronic BBP during a social test displayed increased sniffing and approaching (non-contact behavior). ITI = inter trial interval. Data represent group means ± SEM; *p<0.05

tinguishable (data not shown). Twenty four hours after training on Day 2, rats that were chronically exposed to BBP displayed a decreased freezing behavior in both ITI ($F_{(2,21)}$=5.1; p<0.05) and cue ($F_{(2,20)}$=3.8; p<0.05) phases of fear conditioning by one-way ANOVA, see Figure 2C. Post-hoc tests demonstrated that 10.0 ppm BBP treated rats display decreased rates of freezing compared to 5.0 ppm or VEH (p<0.05).

Social investigative behaviors and the Impact of BBP

We assessed social and approach behavior by in BBP treated rats by using the anosmia model of social behaviors. Figure 2D depicts the increased social interaction with the passive anosmic rat via sniffing and approaching "non-contact" after BBP treatment ($F_{(2,21)}$=3.0; p=0.07) and 10.0 ppm treated rats showing the highest increase (p<0.05).

Biochemical effects of BBP administration

We observed a considerable change in protein levels of MePC2 ($F_{(2,21)}$=9.28; p<0.001) in the amygdala of animals exposed to both 5.0 ppm (p<0.01) 10.0 ppm (p<0.0001) of BBP by one-way ANOVA and post-hoc analysis, see Figure 3A. This was also inversely correlated with anosmic tests of sociability (p<0.01). We observed a considerable decrease in ERK1/2 in the amygdala ($F_{(2,21)}$=3.33; p<0.05) by one-way ANOVA but this was only significant at the highest dose 10.0 ppm (p<0.05), see Figure 3B. We observed no change in stress reactive proteins related to NFkB such as p65 ($F_{(2,21)}$=0.22; p=n.s.) and p50 ($F_{(2,21)}$=0.47; p=n.s.) by one-way ANOVA, see Figure 4A and 4B. Lastly, there were alterations in amygdalar expression of the estrogen receptor α (ERα) ($F_{(2,21)}$=7.08; p<0.005) with an increase in the 5.0 ppm (p<0.05) and a decrease in

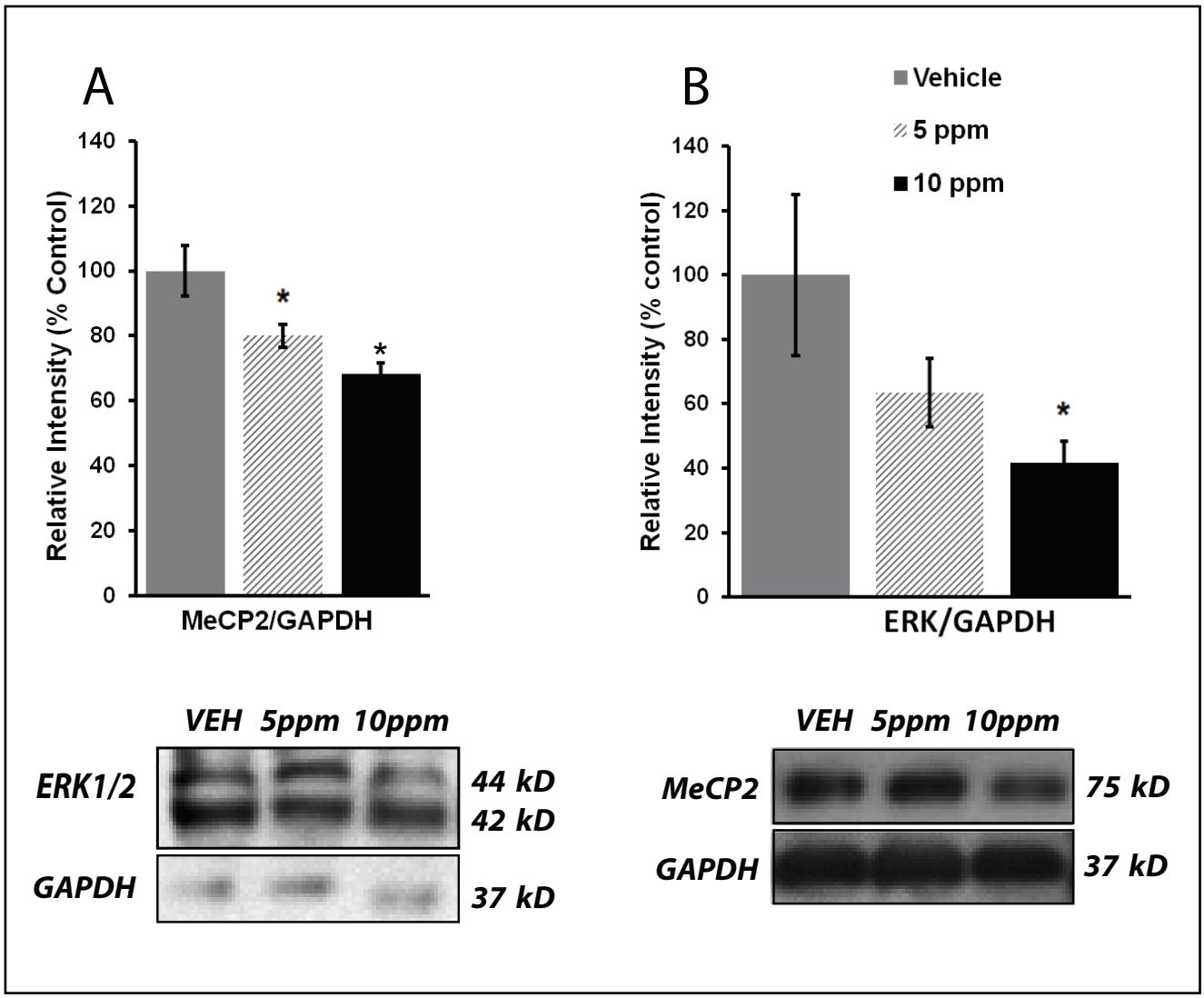


Fig. 3. Chronic BBP treatment can alter protein expression of MeCP2 and ERK1/2 in rats. Fig 3A shows a decrease in amygdalar MeCP2 following BBP treatment. This decrease is inversely correlated aberrant social behavior (p<0.05). Fig 3B shows a decrease in amygdalar ERK1/2 following BBP treatment and is correlated with freezing behavior (p<0.05). All bands were normalized to GAPDH and representative bands from the blots are below the graphical quantification. Data represent group means ± SEM; *p<0.05

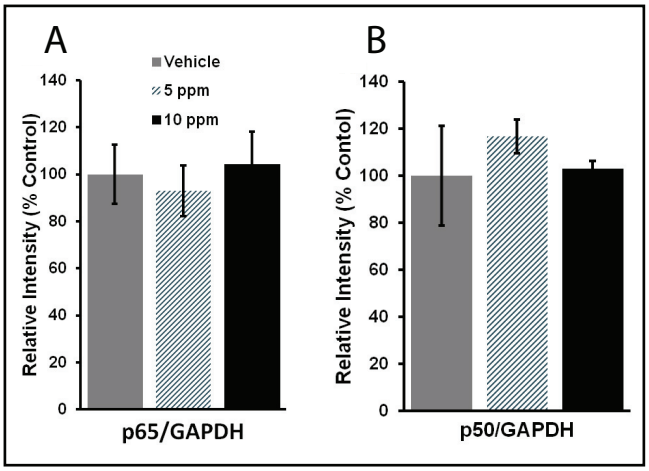


Fig. 4. Proteins related to stress are not affected by chronic BBP treatment in rats. Fig. 4A and 4B show no changes in amygdalar protein expression blotted for p65 and p50 (NFkB subunits) following BBP treatment. There were no significant differences across doses (p=n.s.). Data represent group means ± SEM.

10.0 ppm (p<0.05) by one-way ANOVA and post-hoc analysis.

DISCUSSION

These data presented here are the first to demonstrate that chronic exposure to phthalates can alter the appropriate behavioral response to fear conditioning and social interaction without affecting general activity levels, gross locomotor activity or exploration habits. BBP treated animals also showed abnormalities in protein expression of amygdalar MeCP2, ERα and ERK1/2 but not NFkB subunits.

Physiological and stress responses in BBP treated rats

Rodent toxicology studies suggest that administration of endocrine disruptors produces dysfunctional reproductive and endocrine related responses (Ryan & Vanderbergh 2006). Here, we administered BBP in the drinking water for approximately 15 weeks to juvenile rats. In this experiment, we observed decreases in body weight with no concurrent change in food consumption, indicating that there was a homeostatic imbalance in BBP treated animals. We did not observe changes in adrenal weights, testicular fat or liver weights at the time of sacrifice (data not shown). It is likely that the specific time points of our administration of BBP did not affect the previously developed organs or circulating levels of estrogen. Serum analysis of 17-β estradiol in animals that were administered BBP showed no significant difference. During development, males are extremely sensitive to levels of estrogen; increases in these levels during development can have permanent effects on the development of both reproductive structures leading to a feminization of males and on neuronal development. Future studies will examine the effects of BBP on progeny since changes in estrogen levels during female development can have prolonged effects. Future studies should also examine developmental exposure to phthalates in a temporal fashion. We also observed no protein level changes in NFkB, see Figure 4A and 4B, a critical mediator of stress and depressive behavior (Koo *et al.* 2010). Nevertheless, mechanisms underlying the effects of BBP on stress using corticosterone levels or other physiological markers should be further investigated. Relatively few studies have investigated the link between phthalate exposure and stress.

Use of anosmia as a model of sociability

Rats as a species are social creatures. Two unfamiliar rodents placed together will display several species typical behaviors including sniffing, approaching, contact, and anogenital sniffing (Crawley 2007). A complex task for modeling social behavior and potential aggression is to manipulate the olfactory capacity between unfamiliar rats placed in a neutral setting. Our data demonstrates that after an anosmic and intact BBP treated rat are placed in an arena together, BBP treated rats show dysfunctional responses to the anosmic, passive, rat and display no signs of aggression. Zinc sulfate produces a long lasting, but reversible, anosmic state that is characterized by impaired olfactory discrimination but not the

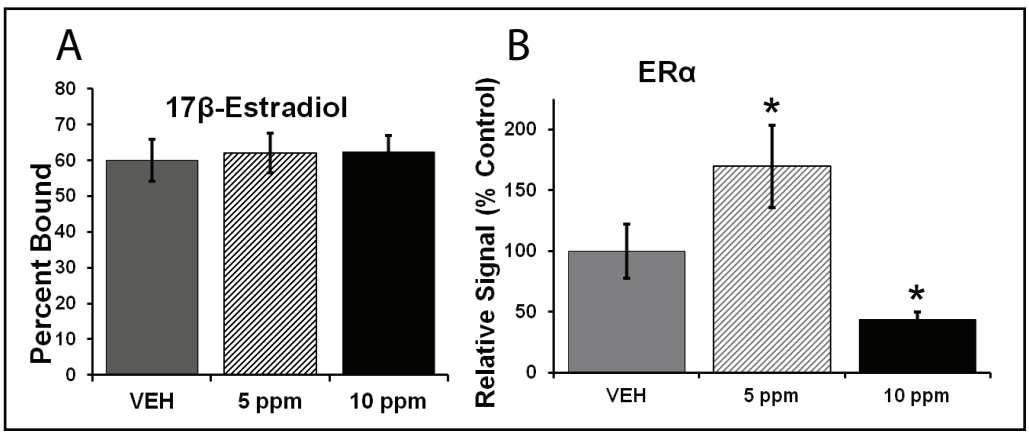


Fig. 5. Fig 5A indicates an increase in amygdalar estrogen α receptor in the 5.0 ppm dose and a decrease in the 10.0 ppm dose. There were no changes in circulating serum 17-β estradiol at the time of sacrifice, Fig. 5B. Data represent group means ± SEM; *p<0.05

ability to sniff. BBP treated rats sniffed and approached the anosmic rats more than control rats. An increase in exploration could explain this result however in tests of open field activity, adult male rats exposed to BBP showed no differences in general ambulation, inner arena crossing, peripheral arena crossings, grooming or rearing. Taken together, this could indicate the rats have an altered sense of novelty seeking or potential anxious state (Crawley 2007). BBP treatment and more sophisticated anxiety testing, such as elevated plus maze, should be performed. Future studies will examine BBP treatment and Crawley's (2007) three chamber sociability and preference for social novelty protocol. While it is possible that we eliminated most of the odors emanating from the anosmic rats it is possible that the BBP treated rats could still respond to the pheromone production of urine (Calvo-Torrent *et al.* 1997). To date, no studies have examined post natal BBP exposure and social responding. There is strong evidence that the epigenetic actions of endocrine disruption can account for behavioral and molecular changes including social behaviors (Dessi-Fulgheri *et al.* 2002; Porrini *et al.* 2005; Cox *et al.* 2010), learning and anxiety (Carr *et al.* 2003; Patisaul and Bateman 2008; Xu *et al.* 2010), sex differences (Farabollini *et al.* 2002; Monje *et al.* 2009), and molecular changes (Tando *et al.* 2007; Tanida *et al.* 2009; Smith *et al.* 2011). Therefore, future studies will examine *in utero* exposure to BBP.

Fear conditioning and amygdalar changes in ERK1/2, ERα and MeCP2 following BBP treatment

Here, a fear memory was established in rats by pairing an environmental stimulus, CS, with a salient aversive US event, foot shock in the fear conditioning paradigm. The strength of this memory was determined by evaluating the CR, freezing, in subsequent tests after retrieval of the original memory event. Our objective was to use this well-established fear conditioning paradigm to understand effects of BBP administration and associative learning. Our data demonstrates that BBP treatment decreases freezing behavior in both the ITI and cue phases of fear conditioning indicating that these rats fail to learn to associate the environment or cue with the shock. Although it is possible that BBP could have altered sensory or pain thresholds, this explanation is unlikely given that freezing on Day 1 training was indistinguishable. Future studies should examine extinction learning after fear conditioning sessions since this is considered a new learning process. A testing sequence such as this would lend insight to the flexibility of their learning ability after BBP treatment.

The neural substrates of fear learning have been well characterized. The amygdalar complex plays a critical role in this defensive behavior, freezing, and has been implicated in the formation of emotional memories. Different sub-nuclei within the amygdala coordinate the expression and execution of fear. It is well known that disruption of the basolateral amygdala affects both acquisition and expression of fear via sensory input (LeDoux *et al.* 1990). It is also generally accepted that the amygdala is critical for learning about both contextual and cued information in the environment (Phillips & LeDoux 1992). Estrogens effects are mediated by ERα and estrogen receptor beta (EBβ) and the amygdala contains a high degree of ERα and there is a strong degree of anatomical distribution of receptor expression between species (Östlund 2003). For this reason, we dissected the amygdala for our biochemical studies. Our results provide further evidence to suggest that estrogens may exert their influence on fear within the amygdala as seen by a decrease in ERα protein coupled with a decrease in freezing behavior in animals exposed to 10.0 ppm BBP.

Future studies should examine the contribution of phthalates exposure and fear circuitry on the hippocampus. We cannot rule out hippocampal contribution in our studies. Although we shifted our context in Day 2 of the fear conditioning paradigm, our freezing data does have contextual, or hippocampal, components to it. Additionally, adult ERα knockout mice have hippocampal dependent memory deficits suggesting that ERα expression is important learning and memory (Foster *et al.* 2008). Nevertheless, this work may reveal a novel and important role of memory processes and ERK1/2 regulation in the amygdala following phthalates exposure. ERK1/2 is a part of the mitogen activated protein kinases (MAPs) family and is a signaling molecules related to learning and plasticity. Additionally, it has been shown that MAPs in the amygdala are required for memory consolidation in fear conditioning. Blockade of ERK1/2 or MAP activation in the lateral amygdala impairs fear memory consolidation (Schafe *et al.* 2000) and long term potentiation (Schafe *et al.* 2008). It is possible that long term exposure to phthalates may dampen this signal cascade and contribute to alterations in memory formation. There is a growing body of work in the literature to showing that activity dependent gene expression mechanisms and epigenetics surrounding memory formation, representation and expression (Nudelman *et al.* 2010; Tronson & Taylor 2007). We demonstrate significantly decreased protein levels of ERK1/2 after chronic BBP administration, and it is important to further explore this interaction and other synaptic plasticity changes in relation to phthalate exposure and fear learning.

Previous studies have shown that other proteins aside from ERK1/2, including MeCP2, a chromosomal protein, in the amygdala is required for normal learning and memory related behaviors (Adachi *et al.* 2009). Several lines of evidence have recently linked MeCP2 function to Rett syndrome, a neurodevelopmental disorder caused by loss of functions in this gene (Amir *et al.* 1999; Chahrour *et al.* 2008; Monteggia *et al.* 2009; Berger-Sweeney 2011). However, its precise role is not fully understood. Rett syndrome and other psychiatric disorders such as autism and Fragile X syndrome are

associated with low levels of social interaction. These disorders are being assessed for translational phenotypes at increasing rates. Knocking down MeCP2 in adult mice produces anxiogenic behavior and cue-dependent fear-conditioning deficits (Adachi *et al.* 2009). Reduced MeCP2 expression is found in the frontal cortex of individuals with autism (Nagarajan *et al.* 2006). In animal studies, MeCP2 is responsible for organizing juvenile social behavior and disruption with small interfering RNA (siRNA) of MeCP2 specifically in the amygdala during sexual differentiation decreased social play in male rats (Kurian *et al.* 2008). MeCP2 null mice, a model of Rett syndrome, display abnormal social responding with other mice (Gemelli *et al.* 2006). Gain of MeCP2 function has also been shown to impair learning and memory and alter synaptic transmission (Na *et al.* 2012). MeCP2 has been indicated as an important contributor during the critical time of brain development due to its role in promoting neuronal growth and maturation. It has been observed that MeCP2 null mice have a decreased brain volume and shorter, underdeveloped dendrite branching compared to controls that may be attributed to an overall decrease in neuronal size (Stearns *et al.* 2007). In the future it would be worth studying the effects of BBP on neuronal growth and maturation in a developing brain to determine if it results in cognitive or learning deficiencies. These lines of evidence point to MeCP2 as a critical mediator of social organization in the brain and its temporal and regional patterns remain uncertain. The complex phenotype associated with Rett syndrome and the nature of the therapeutic window for intervention has yet to be elucidated (Berger-Sweeney 2011). Here, we demonstrate that chronic administration of BBP (10.0 ppm) decreases fear conditioning and it is tightly correlated with decreases in amygdalar MeCP2, ERα and ERK1/2. Taken together, administration of phthalates and endocrine disruption is implicated in hormonal dysregulation which has both behavioral and molecular consequences on the fear and social neurocircuitry.

CONCLUSIONS

Our results indicate an important relationship between aberrant social interactions, fear conditioning (associative learning), and amygdalar MeCP2, ERα and ERK1/2, following BBP administration. Across a range of doses, BBP administration can lead to inappropriate social interaction and disruption of normal maintenance of associative learning. It is highly likely that humans and animals, at all stages of development and beyond, are exposed to phthalates resulting in both behavioral and molecular effects. Given that extensive literature does not exist, it is highly significant to report that phthalate exposure can alter behaviors related to sociability and fear conditioning and that MeCP2, ERα and ERK1/2 in the amygdala may regulate some of these processes. MeCP2-associated disorders, although can be considered distinct, all share core social deficits (Samaco *et al.* 2005) and phthalate exposure should be studied more closely. Taken together, our results suggest BBP exposure has both behavioral and molecular signature. A greater understanding of the consequences of phthalate exposure will translate into more effective interventions for avoidance of this chemical during critical developmental time points. It is possible that multiple mechanisms, multiple routes of exposure and combinations of phthalates together can contribute to the underlying mechanism of social disorders such as autism, Rett syndrome or other psychiatric conditions. The epigenetics surrounding endocrine disruption has yet to be fully elucidated (Wolstenholme *et al.* 2011) and studies such as this play an important role in characterizing the effects of endocrine disruption on the fully developed brain and behavior.

ACKNOWLEDGMENTS

The authors would like to gratefully acknowledge Lisa Kaplan, Douglas Campbell, Renee Rotolo, Ariel Scalise and Nelly Shteynberg for helpful discussions and participation in conducting the experiments. We gratefully acknowledge Dipesh Khanal for his assistance with BBP chemical analysis. We gratefully acknowledge Holli Zampano for her exceptional animal husbandry and help with pilot studies. This research was supported by a grant to AJB from Quinnipiac University and Faculty for Undergraduate Neuroscience.

REFERENCES

1 Adachi M, Autry AE, Covington III HE, Monteggia, LM (2009). MeCP2-Mediated Transcription Repression in the Basolateral Amygdala May Underlie Heightened Anxiety in a Mouse Model of Rett Syndrome. J Neurosci. **29** (13): 4218–4227.
2 Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet. **23**:185–188.
3 Berger-Sweeney JE (2011). Cognitive deficits in Rett syndrome: What we know and what we need to know to treat them. Neurobio of Learn Mem. **96**: 637–646
4 Calafat AM, Ye X, Silva MJ, Kuklenyik Z, Needham LL (2006). Human exposure assessment to environmental chemicals using biomonitoring. Int J Androl. **29**: 166–171.
5 Calvo-Torrent A, Paya-Cano JL and Martinez M (1997). Effect of Anosmia on the Behavior of Standard Non-Aggressive Male Mice Opponents During Agonistic Encounters. Aggressive Behav. **23**: 179–181.
6 Carr R, Bertasi F, Betancourt A, Bowers S, Gandy BS, Ryan P, Willard S. Effect of neonatal rat bisphenol a exposure on performance in the Morris water maze (2003). J Toxicol. Environ Health **66** (21): 2077–2088.
7 Chahrour M, Yun Jung S, Shaw C, Zhou X, Wong STC, Qin J, Zoghbi HY (2008). MeCP2, a Key Contributor to Neurological Disease, Activates and Represses Transcription. Science. **320** (5880): 1224–1229.
8 Cox KH, Gatewood JD, Howeth C, Rissman EF (2010). Gestational exposure to bisphenol A and crossfostering affect behaviors in juvenile mice. Horm Behav. **58** (5): 754–761.

9 Crawley, JN (2007). What's Wrong With My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice. Second Edition. John Wiley and Sons Inc., Hoboken, NJ.
10 Dessi-Fulgheri F, Porrini S, Farabollini F (2002). Effects of perinatal exposure to bisphenol A on play behavior of female and male juvenile rats. Environ Health Perspect. **110** (Suppl 3): 403–407.
11 Duty SM, Singh NP, Silva MJ, Barr DB, Brock JW, Ryan L, Herrick RF, Christiani DC, Hauser R (2003). The Relationship between Environmental Exposures to Phthalates and DNA Damage in Human Sperm Using the Neutral Comet Assay. Environ Health Persp. **111**(9): 1164–1169.
12 Edginton, AN, Ritter L (2008). Predicting plasma concentrations of bisphenol A in children younger than 2 years of age after typical feeding schedules, using a physiologically based toxicokinetic model. Environ Health Perspect. **117**(4): 645–52.
13 Fagin D (2012). The Learning Curve: Researchers Say That Some Chemicals Have Unexpected and Potent Effects at Very Low Doses-but Regulators aren't Convinced. Nature **490**: 462–465.
14 Farabollini F, Porrini S, Della Seta D, Bianchi F, Dessi-Fulgheri F (2002). Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats. Environ Health Perspect. **110** (Suppl 3): 409–414.
15 Foster T, Rani A, Kumar A, Cui L, Semple-Rowland S (2008). Viral Vector–mediated Delivery of Estrogen Receptor-α to the Hippocampus Improves Spatial Learning in Estrogen Receptor-α Knockout Mice. Molecular Therapy. **16**: 1587–1593.
16 Gray EL, Laskey J, Ostby J (2006). Chronic Di-n-butyl Phthalate Exposure in Rats Reduces Fertility and Alters Ovarian Function During Pregnancy in Female Long Evans Hooded Rats. Toxicol Sci. **93**(1): 189–195.
17 Gray EL, Ostby J, Furr J, Price M, Rao Veeramaachaneni DN, Parks L (2000). Perinatal Exposure to the Phthalates DEHP, BBP, and DINP, but Not DEP, DMP, or DOTP, Alters Sexual Differentiation of the Male Rat. Toxicol Sci . **58**(2): 350–365.
18 Gemelli T, Berton O, Nelson ED, Perrotti L, Jaenisch R, Monteggia LM (2006). Postnatal Loss of Methyl-CpG Binding Protein 2 in the Forebrain is Sufficient to Mediate Behavioral Aspects of Rett Syndrome in Mice. Biol Psychiat. **59**: 468–476
19 Hauser R, Meeker JD, Duty S, Silva MJ, Calafat AM (2006). Altered semen quality in relation to urinary concentrations of phthalate monoester and oxidative metabolites. Epidemiology. **17**(6): 682–691.
20 Hauser R, Meeker JD, Singh NP, Silva MJ, Ryan L, Duty S, Calafat AM (2007). DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. Hum Reprod. **22** (3): 688–695.
21 Jang YJ, Park HR, Kim TH, Yang W, Lee J, Choi, SY, Lee, J (2012). High dose bisphenol A impairs hippocampal neurogenesis in female mice across generations. Toxicology. **296**(1–3): 73–82.
22 Koch HM, Drexler H, Angerer, J (2004). Internal exposure of nursery-school children and their parents and teachers to di (2-ethylhexyl) phthalate (DEHP). Int J Hyg and Envir Heal. **207**: 15–22.
23 Koo JW, Russo SJ, Ferguson D, Nestler EJ, Duman RS (2010). Nuclear factor-κB is a critical mediator of stress impaired neurogenesis and depressive behavior. PNAS. **107**(6): 2669–2674.
24 Kurian JR, Bychowski ME, Forbes-Lorman RM, Auger CJ, Auger AP (2008). Mecp2 Organizes Juvenile Social Behavior in a Sex-Specific Manner. J Neurosci. **28** (28): 7137–7142.
25 Lassen C, Maag J, Hubschmann LB, Hansen E, Searl A, Doust E, Corden C (2009). Data on manufacture, import, export, uses and releases of Benzyl butyl phthalate (BBP) as well as information on potential alternatives to its use. COWI, IOM & Entec report to ECHA http://echa.europa.eu/documents/10162/13640/tech_rep_bbp_en.pdf
26 LeDoux JE, Cicchetti P, Xagoraris A, Romanski LM (1990). The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. J Neurosci. **10**: 1062–1069.
27 Lyche JL, Gutleb AC, Berginan A, Eriksen GS, Murk AJ, Ropstad E, Saunders M, Skaare JU (2009). Reproductive and developmental toxicity of phthalates. J Toxicol Environ Health B Crist Rev. **12**: 225–249.
28 Monje L, Varayoud J, Muñoz-de-Toro M, Luque EH, Ramos JG (2009). Neonatal exposure to bisphenol A alters estrogendependent mechanisms governing sexual behavior in the adult female rat. Reprod Toxicol. **28**(4): 435–442.
29 Monteggia LM, Kavalali ET (2009). Rett Syndrome and the Impact of MeCP2 Associated Transcriptional Mechanisms on Neurotransmission. Biol Psychiat. **65**(3): 204–210.
30 Mylchreest E, Wallace DG, Cattley RC, Foster PMD (2000). Dose-Dependent Alterations in Androgen-Regulated Male Reproductive Development in Rats Exposed to Di(n-butyl) Phthalate during Late Gestation. Toxicol Sci. **55**(1): 143–151.
31 Na E, Nelson E, Adachi M, Autry A, Mahgoub M, Kavalali E, Monteggia L (2012). A Mouse Model for MeCP2 Duplication Syndrome: MeCP2 Overexpression Impairs Learning and Memory and Synaptic Transmission. J.Neurosci. **32**(9): 3109–3117.
32 Nagarajan RP, Hogart AR, Gwye Y, Martin MR, LaSalle JM (2006). Reduced MeCP2 expression is frequent in autism frontal cortex and correlates with aberrant MECP2 promoter methylation. Epigenetics. **1**: e1–e11.
33 Nudelman AS, DiRocco DP, Lambert TJ, Garelick MG, Le J, Nathanson NM, Storm DR (2010). Neuronal activity rapidly induces transcription of the CREB-regulated micro-RNA, In vivo. Hippocampus. **20**(4): 492–8.
34 Oehlmann J, Oetken M, Schulte-Oehlmann US (2008). A critical evaluation of the environmental risk assessment for plasticizers in the freshwater environment in Europe, with special emphasis on bisphenol A and endocrine disruption. Environ Research. **108**(2): 140–149.
35 Östlund H, Keller E, & Hurd YL (2003). Estrogen receptor gene expression in relation to neuropsychiatric disorders. Annals New York Acad Sci. **1007**(1): 54–63.
36 Panzica GC, Balthazart J, Frye CA, Garcia-Segura LM, Herbison AE, Mensah-Nyagan AG, et. al. (2011). Milestones on Steroids and the Nervous System: 10 Years of Basic an Translational Research. J Neuroendocrin. **1**: 1–15.
37 Parveen M, Inoue A, Ise R, Tanji M, Kiyama R (2008). Evaluation of estrogenic activity of phthalate esters by gene expression profiling using a focused microarray (Estrarray). Environmental Toxicology and Chemistry. **27**(6): 1416–1425.
38 Patisaul HB, Bateman HL (2008). Neonatal exposure to endocrine active compounds or an ERbeta agonist increases adult anxiety and aggression in gonadally intact male rats. Horm Behav. **53**(4): 580–588.
39 Phillips RG & LeDoux JE (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci. **106**: 274–285.
40 Porrini S, Belloni V, Della Seta D, Farabollini F, Giannelli G, Dessi-Fulgheri F (2005). Early exposure to a low dose of bisphenol A affects socio-sexual behavior of juvenile female rats. Brain Res Bull. **65**(3): 261–266.
41 Richter C, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, Vandenbergh JG, Walser-Kuntz DR, vom Saal F (2007). In vivo effects of bisphenol an in laboratory rodent studies. Reprod Toxicol. **24**(2): 199–224.
42 Ryan BC, Vandenbergh JG (2006). Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice. Horm Behav. **50**: 85–93.
43 Samaco RC, Hogart A, LaSalle JM (2005). Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes reduced expression of *UBE3A* and *GABRB3*. Hum Mol Genet. **14**(4): 483–492.
44 Schafe GE, Atkins CM, Swan MW, Bauer EP, Sweatt JD, LeDoux JE (2000). Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. J Neurosci. **20**(21): 8177–87.
45 Schafe GE, Swank MW, Rodrigues SM, Debiec J, Doyère V (2008). Phosphorylation of ERK/MAP kinase is required for long-term potentiation in anatomically restricted regions of the lateral amygdale in vivo. Learn Mem. **5**(2): 55–62.
46 Silva MJ, Samandar E, Preau JL, Needham LL, Calafat AM (2006). Urinary oxidative metabolites of di(2-ethylhexyl) phthalate in humans. Toxicology. **219**(1–3): 22–23.

47 Smith A, Macdonald A, Holahan MR (2011). Acute postnatal exposure to di(2-ethylhexyl) phthalate adversely impacts hippocampal development in the male rat. Neuroscience. **193**: 100–108C.
48 Stearns NA, Schaevitz LR, Bowling H, Nag N, Berger UV, Berger-Sweeney J (2007). Behavioral and anatomical abnormalities in Mecp2 mutant mice: A model for rett syndrome. Neuroscience. **146**(3): 907–921.
49 Swan SH (2008). Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environ Res. **108**: 177–184.
50 Tando S, Itoh K, Yaoi T, Ikeda J, Fujiwara Y, Fushiki S (2007). Effects of pre- and neonatal exposure to bisphenol A on murine brain development. Brain Dev. **29**(6): 352–356.
51 Tanida T, Warita K, Isihara K, Fukui S, Mitsuhashi T, Sugawara T, Tabuchi Y, Nanmori T, Qi W-M, Inamoto T, Yokoyama T, Kitagawa H, Hoshi N (2009). Fetal and neonatal exposure to three typical environmental chemicals with different mechanisms of action: mixed exposure to phenol, phthalate, and dioxin cancels the effects of sole exposure on mouse midbrain dopaminergic nuclei. Toxicol Lett. **189**(1): 40–47.
52 Tronson NC, Taylor JR (2007). Molecular mechanisms of memory reconsolidation. Nat Rev Neurosci. **8**(4): 262–75.
53 Wolstenholme T, Rissman EF, Connelly JJ (2011). The role of Bisphenol A in shaping the brain, epigenome and behavior. Horm Behav. **59**: 296–305.
54 Xu XH, Zhang J, Wang Y-M, Ye Y-P, Luo Q-Q (2010). Perinatal exposure to bisphenol-A impairs learning memory by concomitant down-regulation of N-methyl-D-aspartate receptors of hippocampus in male offspring mice. Horm Behav. **58**(2): 326–33.