Prenatal Polycyclic Aromatic Hydrocarbon Exposure Leads to Behavioral Deficits and Downregulation of Receptor Tyrosine Kinase, MET


*Department of Neuroscience and Pharmacology, Center for Molecular and Behavioral Neuroscience, Meharry Medical College, Nashville, Tennessee 37208; †Wadsworth Center, New York State Department of Health, Albany, New York 12201-2002; ‡Department of Biochemistry and Cancer Biology, Meharry Medical College, Nashville, Tennessee 37208; §Departments of Pediatrics and Pharmacology, Center in Molecular Toxicology and Kennedy Center for Research on Human Development, Vanderbilt University School of Medicine, Nashville, Tennessee 37212; ¶Department of Psychiatry and the Behavioral Sciences, Zilkha Neurogenetic Institute, Center for Genomic Psychiatry, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089; and ||Department of Cell and Neurobiology, Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, California 90089

1To whom correspondence should be addressed. Fax: (615) 327-6632. E-mail: dhood@mmc.edu.

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Gene by environment interactions (G × E) are thought to underlie neurodevelopmental disorder, etiology, neurodegenerative disorders, including the multiple forms of autism spectrum disorder. However, there is limited biological information, indicating an interaction between specific genes and environmental components. The present study focuses on a major component of airborne pollutants, polycyclic aromatic hydrocarbons (PAHs), such as benzo(a)pyrene [B(a)P], which negatively impacts cognitive development in children who have been exposed in utero. In our study, prenatal exposure of Cprlox/lox timed-pregnant dams to B(a)P (0, 150, 300, and 600 µg/kg body weight via oral gavage) on embryonic day (E14–E17) consistent with our susceptibility-exposure paradigm was combined with the analysis of a replicated autism risk gene, the receptor tyrosine kinase, Met. The results demonstrate a dose-dependent increase in B(a)P metabolite generation in B(a)P-exposed Cprlox/lox offspring. Additionally, a sustained persistence of hydroxy metabolites during the onset of synapse formation was noted, corresponding to the peak of Met expression. Prenatal B(a)P exposure also downregulated Met RNA and protein levels and dysregulated normal temporal patterns of expression during synaptogenesis. Consistent with these data, transcriptional cell-based assays demonstrated that B(a)P exposure directly reduces human MET promoter activity. Furthermore, a functional readout of in utero B(a)P exposure showed a robust reduction in novel object discrimination in B(a)P-exposed Cprlox/lox offspring. These results confirm the notion that common pollutants, such as the PAH B(a)P, can have a direct negative impact on the regulated developmental expression of an autism risk gene with associated negative behavioral learning and memory outcomes.

Key Words: gene × environment interaction; autism spectrum disorders; polycyclic aromatic hydrocarbon; benzo(a)pyrene; susceptibility-exposure paradigm; novel object discrimination behavioral task; in utero exposures; behavioral neurotoxicity.

Although hypotheses that espouse gene by environment (G × E) etiologies for neurodevelopmental disorders are well recognized (Wang et al., 2010), genetic and environmental factors are typically studied separately. For example, replicated genes recently have been identified that increase the risk for autism spectrum disorder (ASD), a highly heterogeneous clinical syndrome in which variation in endophenotypes or disorder expression is likely due to G × E interactions. There is a strong rationale for examining common air pollutants as contributors to putative G × E effects in neurodevelopmental disorders. First, the human health consequences of prenatal exposure to benzo(a)pyrene [B(a)P], a polycyclic aromatic hydrocarbon (PAH), are substantial, with demonstrated associations to reductions in the mental development index and IQ in the high-exposure cohort of 3- and 5-year-old children exposed in utero to PAHs (Perera et al., 2006, 2009). There is a range of B(a)P concentrations to which pregnant women are exposed, which suggests that the cognitive impact on their fetuses is due to a more complex mechanism than the well-described aryl hydrocarbon receptor signaling (Nebert et al., 2000). Brain levels of PAHs in B(a)P-exposed children cannot be monitored, thus requiring more mechanistic studies utilizing mouse models to define developmental neurobiological effects resulting from exposure to PAHs during key periods of circuit formation in the brain. Moreover, this strategy provides opportunities to directly examine prenatal PAH impact on recognized candidate genes for specific neurodevelopmental disorders.

The objective of the present study was to characterize the interaction between in utero exposure to B(a)P and the gene encoding the MET receptor tyrosine kinase, a replicated ASD risk gene (Jackson et al., 2009; Sousa et al., 2009) that mediates neuronal process growth and synapse formation (Akita et al., 2008; Gutierrez et al., 2004; Nakano et al., 2007). Our own evaluation of linkage peaks extended beyond genes...
with selective brain expression to consider the complex medical conditions observed in autism patients. Over the years, analytical applications in developmental biology and genetics have converged to implicate the gene encoding the MET receptor tyrosine kinase (OMIM 164860; GenBank accession NM_000245; chromosome 7q31) as an autism candidate gene. Recent studies by the Levitt group and others have revealed that MET also contributes to the development of the cerebral cortex (Powell et al., 2001, 2003), which exhibits developmental disruptions in autism (Palmen et al., 2004) and in animal model susceptibility paradigms of developmental environmental contaminant exposure (Hood et al., 2006; McCallister et al., 2008).

The relationship between genetic risk and phenotypes characteristic of ASDs lies in the patterns of gene expression and the function of the encoded proteins in specific circuits during development. Based on recent biological studies, we have suggested that MET receptor activation influences the development and maturation of these circuits (Levitt and Campbell, 2009). This neurodevelopmental role is consistent with recent genetic findings that indicate a significant association of the MET promoter variant rs1858830 C allele with ASD risk in three independent cohorts (Campbell et al., 2006, 2008). Furthermore, MET transcript and MET protein have been found to be significantly decreased in postmortem brains of individuals with ASD (Campbell et al., 2007).

Our focus on MET receptor tyrosine kinase is justified on the basis of relevant human studies, and it is likely only one of the number of targets for B(a)P. Met expression in the mouse coincides with the onset and peak of synapse formation in the neocortex during the first two postnatal weeks (Judson et al., 2009). In the human, this corresponds to an exposure period during the second and third trimester and expression analysis in third trimester through the first postnatal year (Levitt, 2003). Genetically induced hypomorphic Met signaling alters neuronal differentiation, and stimulation of the receptor by its sole ligand, hepatocyte growth factor, enhances the expression of NMDA receptor subunits (Tyndall and Walikonis, 2006). PAHs have been shown to alter developmental processes involving glutamatergic signaling (McCallister et al., 2008, Wormley et al., 2004). Here, we used a mouse model of in utero exposure, similar in design to those of other rodents, with a focus on Met in order to establish parameters for the direct examination of G × E effects that may be relevant to a specific subset of environmental-induced ASD-like symptomology.

Our wild-type (WT) Cpr mouse model is but one of the many such models. Mutant P-450 oxidoreductase (POR) alleles have been found to be associated with congenital deficiencies in steroidogenesis/homeostasis and/or with the Antley Bixler Syndrome, characterized by skeletal malformation and reproductive defects (Fluck et al., 2004; Fukami et al., 2005). Other known phenotypes of the Cpr-low mouse, such as lower cholesterol levels and reduced embryonic survival, as well as phenotypes yet to be identified, are also anticipated to occur in human patients. The results from our study demonstrate the functional impact of in utero B(a)P exposure as tested by examining the impact on behavior that is mediated by maturing cortical circuits.

MATERIALS AND METHODS

The WT mouse utilized and characterized in the present study is the Cytochrome p450 reductase or Cprlox/lox mouse. Nested in this study is an obligatory characterization of this WT Cprlox/lox mouse for B(a)P disposition and metabolite bioavailability against the background C57BL/6J strain. The usefulness of this model and its relevance to human POR (POR; for P450 oxidoreductase) deficiency has been widely reported and is supported by the occurrence of substantial interindividual variations in levels of Cpr expression in human tissues (Wortham et al., 2007).

Utilization of the Cpr mouse model will be important for future studies in which a Cpr-low activity (i.e., brain-Cpr-null) phenotype has been identified (Gu et al., 2007). This brain-Cpr-null phenotype does not possess the ability to produce B(a)P metabolites from the parent compound due to the reduced expression of POR, thus allowing determinations of the extent to which oxidative metabolites of B(a)P contribute to any observed developmental and behavioral defects.

Power analysis was used to determine the appropriate number of Cprlox/lox offspring required; the number of litters was used as the statistical unit. For the present analysis, four cohorts of four experimental groups were utilized: (1) a control Cprlox/lox group (vehicle exposed with peanut oil); (2) a 150 µg/kg body weight (BW) B(a)P-exposed Cprlox/lox group; (3) a 300 µg/kg BW B(a)P-exposed Cprlox/lox group; and (4) a 600 µg/kg BW B(a)P-exposed Cprlox/lox group. All experiments utilized groups 1 through 3 except for the semi-quantitative PCR experiments. Cprlox/lox offspring mice from control (n = 4–5 litters) and B(a)P-exposed (n = 4–5 litters) dams were used to collect the molecular and behavioral data. For each repetition, it was estimated that the variance between measures from litters would be 10% of the mean response. Because the litter was the statistical unit, three replicates for metabolite disposition and molecular and behavioral studies were deemed to be sufficient because the power analysis established that three successful experiments in each Cprlox/lox cohort would be required to detect a 20% change in any of the experimental end points with 80% power and a type I error rate of 5%. This estimate held true as we were able to detect differences ± the variance in the effect of B(a)P exposure on Cprlox/lox offspring relative to control offspring for metabolic disposition, molecular and behavioral studies.

Animal husbandry and B(a)P treatment using the susceptibility-exposure paradigm. Experiments were approved by the Institutional Animal Care and Use Committee of Meharry Medical College and were performed according to Guidelines for Animal Experimentation as set forth by the National Institutes of Health and both institutions. Cprlox/lox mice were obtained from the Wadsworth Center, New York State Department of Health, Albany, New York. Cprlox/lox mating pairs were maintained in our AAALAC accredited animal care facility and are propagated to maintain our female F1 generation colony to ~30 breeders. Typically, a male of the appropriate genotype was placed with a pair of females (3–4 months old) of the appropriate genotype overnight, 1× per week. During this period, females received a nutritional high-fat diet supplement (Lovenash Bioserv, Frenchtown, NJ) that has proven helpful in stabilizing litter size. A combination of a positive sperm plug, positive vaginal smear identification, and the monitoring of weight gain ensured the identification of pregnant Cprlox/lox dams by E11. Subsequently, four to five timed-pregnant Cprlox/lox females per group were moved into a 3-day holding protocol prior to B(a)P exposure.

On embryonic day (E14–E17), Cprlox/lox dams were exposed to B(a)P (150, 300, and 600 µg/kg BW) by oral gavage. The proper controls for the B(a)P (in peanut oil) gavage exposures of Cprlox/lox timed-pregnant dams were, in fact,
conducted. These controls consisted of Cprlox/lox timed-pregnant dams that were sham exposed to an equivalent volume of peanut oil. Generally, Cprlox/lox dams gave birth on E20. Following delivery, Cprlox/lox dams were supplemented with Supreme Mini-Treats (Bioserv, Frenchtown, NJ) to reduce cannibalism. Cprlox/lox litters were maintained in a controlled environment with a temperature set at 21 ± 2°C and a relative humidity of 50 ± 10% with a 12/12 h-light/dark cycle. Cprlox/lox dams were fed commercial food (Rat Chow 5012; Purina Mills, St Louis, MO). Water and food were available ad libitum. The numbers of pups within the control and treatment groups were not standardized on the day of birth due to the need to remove pups from litters to conduct metabolite disposition and expression profiling studies. Litter size and pup weights were recorded. On various postnatal days (PND), pups were removed from litters and anesthetized with CO2. For PND 0–5, whole brains were exposed, removed, and frozen in liquid nitrogen until the homogenates were prepared for B(a)P metabolite disposition and mRNA and protein expression profiling analysis. For PND 7–15, left and right cerebral cortices were exposed, removed, and frozen in liquid nitrogen until the homogenates were prepared for B(a)P metabolite disposition and mRNA and protein expression profiling analysis. Additionally, at autopsies, a 0.25- to 0.35-cm piece of the tail was collected for genotyping. Each data point for metabolite disposition and expression profiling comprised 100–200 mg portions of the whole brain (for P0–P5 time points) or 100–200 mg portions of the left or right cerebral cortex (for P7–P15 time points) from at least four to five different litters within the experimental groups.

**B(a)P metabolite disposition and analysis from whole-brain or cerebral cortex tissue.** Whole-brain tissue or left-right cerebral cortices from control and B(a)P-exposed Cprlox/lox offspring were processed for bioavailable levels of B(a)P metabolites by liquid-liquid extraction and reverse phase high-performance liquid chromatography methods as described previously in Ramesh et al. (2001).

**Cell transcription assays.** The cell-based transcriptional assays were performed as described previously (Campbell et al., 2006) using a 762-bp fragment of the MET promoter, which encompasses the rs1858830 locus. Following the manufacturer’s protocols, the human MET amplicon was cloned into the pSTBlue-1 AccepTor vector (Novagen, Madison, WI), which was then directionally subcloned into the pGL4.10 (luc2) luciferase reporter vector (Promega, Madison, WI). Human embryonic kidney (HEK) cell lines (ATCC) were also used. For exposure experiments, 2 μg of luciferase reporter construct were cotransfected with 80 ng of pRL-CMV (Renilla luciferase fused to the strong cytomegalovirus promoter; Promega) using the Amaxa Nucleofector system (Amaxa, Gaithersburg, MD). B(a)P (98% pure; Sigma) was resuspended in 100% dimethyl sulfoxide (DMSO) at a concentration of 2mM. Serial dilutions were prepared in 100% DMSO. Twenty-four hours after transfection, cells were exposed to a range of 100–1000nM B(a)P or DMSO vehicle (0.05%) in Dulbecco’s Modified Eagle’s Medium growth medium. Luciferase assays were conducted using the Dual-Glo Luciferase Assay Kit (Promega) according to the manufacturer’s protocol. Each transfection was represented by six luminescence readings for both firefly and Renilla luciferase. The average luminescence reading from the mock-transfected negative control was used for the background and was subtracted from each well. The ratio of experimental (firefly) to control (Renilla) luciferase was calculated, and the six repeated ratios of each condition were averaged to give a single experimental/control ratio for each transfection. The experimental/control ratio of each MET promoter-containing construct, in nonexposed and chemical-exposed samples, was calculated. Data are expressed as the fold induction over promoter-less vector. Each transfection was considered a separate sample, and each condition was repeated six times (n = 6). ANOVA followed by the Dunnett’s t-test was used to compare each concentration of B(a)P to the nontoxicant control.

**RT-PCR and Western analysis for mRNA/protein content of Met in brain tissue.** To detect B(a)P-induced temporal modulation of Met mRNA and protein expression in control and B(a)P-exposed Cprlox/lox offspring, semiquantitative RT-PCR and Western blot analyses were used. Both complementary DNA synthesis and RT-PCR amplification were produced in one step using Promega’s Access RT-PCR System; the sequences of designed primers and the final concentrations of reagents in the PCRs system were as previously indicated (McCallister et al., 2008). Target gene mRNA expression is presented as the relative ratio between the intensity of the target-specific band to that of the 18sRNA band. For protein evaluation, aliquots (50–100 μg) of the pooled littermate Cprlox/lox offspring cerebral cortices were used. Samples were lysed by homogenization in ice-cold buffer containing 50mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.4), 150mM NaCl, 20mM EDTA, 100μM NaF, 10mM Na3VO4, 1mM phenylmethylsulfonyl fluoride, 1mM leupeptin, 20 μg/ml aprotinin, and 1% Nonidet P-40. The protein content of the supernatants was determined using Bio-Rad protein assay reagent. Equal amounts of protein were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and Met was detected with specific antibodies. After incubation with mouse anti-Met primary antibody (Santa Cruz #8057), the nitrocellulose membranes were incubated with donkey anti-mouse peroxidase-conjugated secondary antibody. Proteins were visualized with the Amersham enhanced chemiluminescence system.

**Statistical analysis of developmental expression profiles (mRNA and protein).** The values for Met mRNA and protein are reported as means ± SEM. A two-way ANOVA was used for the determination of statistical differences between control and B(a)P-exposed Cprlox/lox offspring groups in target mRNA gene/protein expression levels relative to 18sRNA/β-actin. The 18s was used as an internal standard for the RT-PCR analysis because this gene is not modulated as a result of B(a)P exposure. The 18s comprises up to 80% of a total RNA sample; thus, when the concentration of a total RNA sample is determined from spectrophotometric readings, the sample is essentially already normalized to the amount of rRNA it contains. RNAs also are transcribed by a polymerase distinct from that of mRNAs, which may result in a different pattern of regulatory expression. The 18s rRNAs have been found to be uniform in all rodent tissues tested, including the liver, brain, thymus, heart, lung, spleen, testes, ovary, kidney, and in the embryo. Therefore, the choice of a housekeeping gene in this neurotoxicity study is based on empirical determinations regarding the effect of the toxicant on the system under examination. All pairwise multiple comparisons were performed using the Student-Newman-Keuls method. The criterion for statistical significance was p < 0.05 in all cases.

**Novel object discrimination.** On P40, each mouse underwent one 30-min habituation session in a square activity chamber (43.2 × 43.2 cm; Med Associates, St Albans, VT). The automated activity chamber monitored the horizontal and vertical movement of the mouse by quantifying the photo-beam disruption. Following this habituation, the mouse was returned to its home cage for 24 h, after which each animal underwent four habituation and testing sessions, each 15 min long, separated by 15 min-intervals. In the first session, the mouse was allowed to habituate to the empty activity chamber for 30 min. In session 2, the animal was allowed to familiarize itself with two objects (familiar) placed in the rear chamber for 6 min. In session 3, the mouse was again placed in the activity chamber with the same arrangement of objects. Finally, in session 4, the session that tested response to novelty, one of the familiar objects from the previous session was replaced with a novel object for 6 min. Both the presentation and position of objects were alternated as a control.

**Analysis of responses to novelty in control and B(a)P-exposed Cprlox/lox offspring.** Using open field activity software (Med Associates), activity in the chamber was measured. This software allows for post hoc definition of zones of interest. A zone of any size can be defined by selecting grid squares of photo-beam intersections. With the open field activity interface, a small zone (5 × 5 photo-beam grid) and a larger zone (6 × 6 photo-beam grid) were designated around each object. The software collected and documented the total activity, number of entries into each zone (object approaches), and the time spent in each zone. Simultaneously, zones of interest were designated around each object. The software recorded using a stopwatch blinded to the identity of the experimental groups. Observational time included only that time during which...
the animal was in close proximity (within 2 cm) or actively touching or sniffing the object. The novelty index was calculated using the formula $NI = (n - f)/(n + f)$, where $n$ is the time with the novel object and $f$ is the time with the familiar object. This index ranges from $-1$ to $1$, with $a = -1$ signifying complete preference for the familiar object, $0$ signifying no preference, and $1$ signifying complete preference for the novel object. Statistical evaluations were made using ANOVA and planned comparisons. An $\alpha$ level of 0.05 was considered significant for all statistical tests employed.

RESULTS

The $Cpr^{lox/lox}$ mouse, a transgenic mouse strain generated for the conditional deletion of the NADPH-cytochrome P450 reductase ($Cpr$) gene, is essentially identical to the WT mouse, as the targeted insertion of the two loxP sites into the $Cpr$ locus does not cause any effects on $Cpr$ gene expression (Wu et al., 2003). The mouse, originally produced on a mixed C57BL/6 and 129/Sv background, has been backcrossed to the C57BL/6 strain for over 10 generations. As mentioned in the “Materials and Methods” section, the $Cpr^{lox/lox}$ mouse is the WT control strain for future studies that will utilize tissue-specific $Cpr$-null mouse models, such as brain-$Cpr$-null (Conroy et al., 2010) and liver-$Cpr$-null models (Gu et al., 2003).

Using $Cpr^{lox/lox}$ mice in these studies, an analysis of live birth indices revealed no significant differences in the number of mouse pups born per litter between control $Cpr^{lox/lox}$ dams and $B(a)P$-exposed $Cpr^{lox/lox}$ dams (150, 300, and 600 $\mu$g/kg BW). A hallmark of the susceptibility-exposure paradigm is that the $B(a)P$ exposure is restricted to a time window (E14–E17) that occurs well after implantation on E12 so as not to adversely affect fetus’ on the uterine horn. The live birth index for control $Cpr^{lox/lox}$ dams was $5.22 \pm 0.88$ compared with $6.5 \pm 0.69$ for $B(a)P$-exposed $Cpr^{lox/lox}$ dams ($p = 0.255$). These normal parameters are consistent with previous reports from our group (Brown et al., 2007; Hood et al., 2000; McCallister et al., 2008) using rat models. During the prenatal exposure and subsequent preweaning periods, there were no identifiable $B(a)P$-related effects on conventional reproductive indices of toxicity. Additionally, there were no convulsions, tremors, or abnormal movements noted in any of the dams or offspring in control or $B(a)P$-exposed litters.

Figure 1A provides data demonstrating the similarity of $POR$ activity as assessed by the distribution of total $B(a)P$ metabolites in the C57BL/6 timed-pregnant dam on PND 3 exposed to 600 $\mu$g/kg BW $B(a)P$ from E14–E17. Inspection of Figure 1A reveals that on PND 3, the 4,5 diol groups of $B(a)P$ metabolites comprise ~80% of the total metabolite distribution in C57BL/6 dams that were exposed to $B(a)P$ from E14–E17. Comparatively, the disposition in $Cpr^{lox/lox}$ offspring (PND 3) that were exposed in utero to 600 $\mu$g/kg BW $B(a)P$ (Fig. 1B) comprise ~80% of the total metabolite distribution in these $B(a)P$-exposed $Cpr^{lox/lox}$ offspring. The individual metabolites present in the $B(a)P$-exposed C57BL dams and $Cpr^{lox/lox}$ offspring included the 4,5-,7,8,-9,10-diols, 3,6, and 6, 12-diones and 3-OH and 9-OH $B(a)P$ metabolites. Collectively, these data provide sufficient evidence to conclude that the $POR$ are functioning at comparable rates in the WT strain of our mouse model as compared with the background C57BL strain. Figure 1B shows the dose-dependent accumulation (150, 300, or 600 $\mu$g/kg BW) of total $B(a)P$ metabolites in neocortex during the critical postnatal period when synapses are first formed in $B(a)P$-exposed $Cpr^{lox/lox}$ offspring. Data are shown for total neocortical $B(a)P$ metabolite concentrations derived from $Cpr^{lox/lox}$ offspring on PND 3, 5, 10, 15, and 20. On PND 3, a clear dose-response for $B(a)P$ accumulation in neocortex is evident and indicative of an active phase 1 biotransformation pathway in our $Cpr^{lox/lox}$ mouse model. Given the results of these disposition studies, it is important to emphasize here that the neocortical tissue burden of metabolites resulting from a 600 $\mu$g/kg oral exposure is less than the dietary intake of $B(a)P$ by humans. This mean dietary intake of $B(a)P$ by humans is 2–500 ng/day (Liyi et al., 1988) which translates to 200–7000 $\mu$g/kg/day for a healthy 70 kg individual.

A comparison of $Met$ transcript expression in the cerebral cortex at the onset (birth) through the most rapid production phase (PND 7) of synapses in control $Cpr^{lox/lox}$ and $B(a)P$-exposed $Cpr^{lox/lox}$ offspring is shown in Figure 2. Although there are no differences in $Met$ expression at birth for any exposure dose, a statistically significant reduction in $Met$ transcript is observed from PND 1 through 7 at the middle (300 $\mu$g/kg BW) and high (600 $\mu$g/kg BW) $B(a)P$ doses used in the study. The results suggest that $B(a)P$ exposure can reduce $Met$ gene transcription directly. In order to corroborate the fact that $B(a)P$ exposure alone is sufficient to alter MET expression, in vitro cell-based transcription assays were performed as described previously using the 762-bp fragment of the MET promoter variant rs1858830 locus allele, “C” or “G”. HEK cells were transfected with the luciferase reporter constructs containing 762 bp of the MET promoter including either the “C” or “G” allele at the rs1858830 variant locus. Twenty-four hours after transfection, the cells were exposed for an additional 24 h to concentrations of $B(a)P$ as indicated in Figure 3. These data clearly demonstrate a greater than fivefold significant decrease in MET transcription as a function of $B(a)P$ exposure concentration. The data also illustrate the utility of luciferase assays to quantify the relative levels of MET promoter-driven transcription in our model. Although 500nM was not the highest concentration used in the study, from an environmental exposure standpoint, this amount represents an environmentally relevant concentration for $B(a)P$. These findings support the semiquantitative PCR results which demonstrate the $B(a)P$-induced downregulation of specific postnatal period (PND 1 through 14) following in utero
FIG. 1. B(a)P metabolite disposition during postnatal brain/cortical development. (A) Distribution of metabolite types in B(a)P-exposed C57BL/6 timed-pregnant dams. C57BL timed-pregnant dams received 600 μg B(a)P/kg BW via oral gavage on E14–E17. Neocortical tissue was retrieved from B(a)P-exposed C57BL dam on PND 3 and the distribution of the individual B(a)P metabolites was determined by high-performance liquid chromatography (HPLC) as outlined in Ramesh et al. (2001). Values represent mean ± SEM. The asterisk denotes statistical significance (p < 0.001) for different metabolite concentrations as compared with the 4,5-diol metabolite. (B) Distribution of metabolite types in B(a)P-exposed Cprlox/lox offspring demonstrating comparable POR activity in brain tissue. Timed-pregnant Cprlox/lox dams received 600 μg B(a)P/kg BW via oral gavage on E14–E17. Neocortical tissue was retrieved from B(a)P-exposed Cprlox/lox offspring on PND 3, and the distribution of the individual B(a)P metabolites was determined by HPLC as outlined in Ramesh et al. (2001). Values represent mean ± SEM. The asterisk denotes statistical significance (p < 0.001) for different metabolite concentrations as compared with the 4,5-diol metabolite. (C) Dose-dependent accumulation of total neocortical B(a)P metabolites during the critical postnatal period when synapses are first formed in B(a)P-exposed Cprlox/lox offspring. Cprlox/lox offspring dosed in utero with either 150, 300, or 600 μg/kg BW were sacrificed on the indicated postnatal day, and brain tissue was frozen in liquid nitrogen subsequent to genotyping on PND 20. Offspring pups that were identified as carrying the allele Cprlox/lox were pooled, extracted, and the B(a)P metabolite concentrations was determined by HPLC as outlined in Ramesh et al. (2001). Shown are total metabolite concentrations measured in B(a)P-exposed Cprlox/lox offspring for the three B(a)P doses. Values represent mean ± SEM. Due to the limited volume of neocortical tissue present on PND 3 and 5, whole-brain tissue was used for these time points. The asterisk denotes statistical significance at p < 0.05.
first attends to two identical familiar objects. Upon replacement of one of the familiar objects with a novel object, the animal should display preferential behavior toward the novel object. This generally occurs if the mouse can recognize that the object is novel. It is readily apparent in Figure 5 that each control Cprlox/lox offspring mouse tested spent more time with the novel than the familiar object, thereby producing a positive mean novelty discrimination index of 0.54 ± 0.13. Zones of two sizes (novel and familiar object; see Fig. 5) were quantitated around the novel and familiar objects in order to compare the observational data. An analysis of variance revealed no significant differences between the five control Cprlox/lox offspring (observational, small zone, and large zone) with regard to novelty index data. However, for the five B(a)P-exposed Cprlox/lox offspring tested, significantly less time was spent with the novel than the familiar object as compared with controls, thereby producing a negative mean novelty discrimination index score of −0.26 ± 0.07 (p < 0.05 for the 150 µg/kg BW group). For the five B(a)P-exposed Cprlox/lox offspring in the 300 µg/kg BW group, the statistical significance of the deficit in response to novelty was even greater at −0.23 ± 0.10. Post hoc analysis with the Bonferroni test revealed the significance at p < 0.01.

**DISCUSSION**

The present study establishes the validity of using genetically tractable mouse models (including the Cprlox/lox model) to explore G × E influences on molecular, structural, and functional brain development. Going forward, many of the Cpr phenotypes that we plan to exploit have already been identified including the Cpr-low mouse that exhibits a degree of infertility and altered steroid homeostasis (Wu et al., 2007).
The compelling rationale for the receptor tyrosine kinase, MET, as a viable target for gene × environment interactions during critical periods of development is based on a recently reported study on ASD (Morrow et al., 2008). The results from the current study have offered valuable data, clarifying our overarching hypothesis relative to the regulation of gene expression in neuronal membrane depolarization, which we believe may also be negatively impacted in ASD. Our hypothesis suggests that the environmental B(a)P exposure-induced downregulation of the neuronal activity-dependent regulation of synapse development is a mechanism that is commonly found in environmental exposure-induced ASD subtypes. We know that postnatal brain development does require input from the environment in order to induce the release of glutamate and thereby promote critical aspects of synaptic maturation. It is during this process of postnatal synaptogenesis that the effects of in utero B(a)P exposure on neural activity are most likely to alter the expression of genes, each with its unique temporal expression profile. Moreover, prenatal exposure for a restricted time period is sufficient to generate long-term changes in Met expression after birth, which in conjunction with other molecular disruptions, are manifested in significant behavioral deficits. B(a)P exposure at 150 μg/kg resulted in reductions in Met expression at PND 10 and 15. A 300 μg/kg exposure resulted in reductions in Met expression at all PNDs examined. Although the lower dose could represent a developmental shift in expression pattern, we believe that the reduction at multiple postnatal time points is consistent with an interpretation that B(a)P exposure at certain prenatal concentrations is sufficient to reduce Met expression throughout postnatal development. Given the role of Met in dendrite and spine growth (Judson et al., 2009), a reduction or shift during the period of peak synapse formation may result in altered maturation of cortical connections. Although it is likely that a number of genes are targeted by B(a)P, the alteration in Met expression would be consistent with its contribution to the disruption of complex behaviors tested here.

The paradigm of B(a)P exposure during pregnancy used in this study mimics the kinetics of accumulation in brain tissues measured in other rodent species (Brown et al., 2007; Hood et al., 2000; McCallister et al., 2008; Ramesh et al., 2001), which likely is due to transfer via both placental transport and lactation. Extrapolating from the results reported here as well as those from previous experiments in rats (Brown et al., 2007; Hood et al., 2000; McCallister et al., 2008), it appears that prenatal B(a)P exposure can have a robust negative impact on key molecular constituents that are important for the development of excitatory synapses. These B(a)P exposure–induced alterations suggest that G × E interactions can be examined directly in mouse models by imposing both genetic and environmental loads that may exacerbate the impact of each individual component on cortical development and cognitive behaviors that are relevant to understanding the G × E impact in clinical populations.
The focus on understanding the impact of exposure to a common environmental pollutant on MET is based in part on our initial discovery (Campbell et al., 2006), which was subsequently replicated (Campbell et al., 2009; Jackson et al., 2009; Judson et al., 2009), of MET as an ASD risk gene. Moreover, one of the ASD risk alleles, located in the proximal MET promoter, was shown to alter nuclear protein binding, including that of Sp family members (Campbell et al., 2006). This finding is of significant interest because B(a)P exposure in rats has been shown to affect the temporal expression of neuronal Sp proteins that regulate downstream genes critical to circuit integrity, demonstrates a significant negative impact by described in this study as a measure of prefrontal and limbic thalamocortical synaptic drive and disruptions in primary sensory consequences which are manifested by perturbations in the hippocampus. B(a)P exposure in utero has a more pervasive impact on cortical circuits, which could be due in part to the disruption of genes regulated by Sp family members (Liu et al., 2003).

We evaluated Cprlox/lox offspring in a behavioral paradigm that focuses on attention, learning and memory impairments, and responses to novelty, all of which are relevant functional domains that exhibit varying degrees of dysfunction in individuals with ASD (Geschwind, 2009). The novel object discrimination task was successfully used in this study to demonstrate alterations in novelty processing in Cprlox/lox offspring as a function of in utero B(a)P exposure. Prenatal exposure to B(a)P was shown to significantly disrupt performance in this task in B(a)P-exposed Cprlox/lox offspring. The results from these studies suggest that the novel object discrimination task is sensitive enough to detect deficits in response to novelty that are reflective of attention and learning and memory impediments (Fig. 5). Collectively, the results presented in the present study strongly suggest that it is highly likely that environmental factors and/or a combination of genetic variants may underlie the heterogeneity of behavioral phenotypes that, in part, determine neurobehavioral pathology. Finally, we have chosen, in this instance, to illustrate the environmental relevance of the highest dose of B(a)P used in the present study (600 mg/kg BW) within the context of neocortical tissue burden during development. As an additional point of reference to the Perera study (Perera et al., 2009), we reference the neocortical tissue burden resulting from a B(a)P aerosol concentration of 100 µg/m³ (Hood et al., 2000). The significance of the developmental neocortical tissue metabolite burden can then be appreciated within the context of the data reported in Perera et al. (2009). In that study, 5-year-old children were exposed in utero to 0.49–34.48 ng/m³ PAHs where greater than half of children ended up in the high PAH exposure group. When these children were tested at 12, 24, and 36 months, children in the high-exposure group had significantly lower full-scale/verbal IQ scores than the low-PAH exposure group. A very recent report by Wang et al. (2010) has reported the identification of the gene × environment interaction between prenatal PAH exposure and a haplotype of CYP1B1 gene (ACCGGC) responsible for the decline in mental development index from that cohort of children.

The utility, relevance, and impact of the present study are grounded in the fact that these observations have now been replicated in a transgenic mouse model that is analogous to the human condition. In humans, cytochrome P450-1B1 is the primary extrahepatic PAH bioactivating enzyme in developing neocortical tissue. Our WT Cprlox/lox mouse has proved to be an ideal model to assess the contribution of P450 enzymes to the metabolic activation and disposition of environmental xenobiotics. This is evidenced by the fact that the actual xenobiotic metabolite disposition to neocortex in the present study is well within the order of magnitude of the predicted disposition to the fetus for the environmental concentrations for PAHs that were reported in Perera et al. (2009) and Wang et al. (2010).

In conclusion, specific developmental events such as glutamatergic excitatory synapse formation and maturation may be particularly vulnerable to G × E effects that impact regulatory and signaling proteins involved in this process. Although we do not suggest that the current study reflects specific defects related to a complex clinical condition such as the ASDs, current molecular, behavioral, and functional imaging data are converging on the concept that the ASDs are a manifestation of altered local and long-distance cortical connectivity (Bill and Geschwind, 2009; Geschwind and Levitt, 2007; Levitt and Campbell, 2009). Also, Met and other related signaling components of this receptor tyrosine kinase pathway have been implicated in both syndromic and idiopathic disorders where the ASDs are diagnosed at a high rate. In combination with risk alleles in key genes, the in utero exposure to PAHs such as B(a)P, which results in both a reduction in absolute levels and the mistiming of peak Met expression, could drive the system toward a pathophysiological threshold that neither genetic risk nor environmental factors could produce individually (Campbell et al., 2010). The present study focused on the neocortex, but given the highly restricted spatial and temporal expression of Met in mouse limbic circuits associated with social-emotional development and cognition (Judson et al., 2009), it is likely that perturbations occur throughout these key circuits, including in the hippocampus. Studies in the immediate future will seek to elucidate the functional changes and specific molecular mechanisms underlying alterations in Met-driven neural activity and plasticity using this G × E model.

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