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Downregulation of Early Ionotropic Glutamate Receptor Subunit Developmental Expression as a Mechanism for Observed Plasticity Deficits Following Gestational Exposure to Benzo(a)pyrene

La'Nissa A. Brown¹, Habibeh Khoshbouei¹, J. Shawn Goodwin^{1,Π}, Charletha V. Irvin-Wilson¹, Aramandla Ramesh^{1,Π}, Liu Sheng¹, Monique M. McCallister¹, George C. T. Jiang², Michael Aschner², and Darryl B. Hood^{1*}

¹Department of Neurobiology and Neurotoxicology, Center for Molecular and Behavioral Neuroscience, Meharry Medical College, Nashville, TN 37208

^ΠDepartment of Cancer Biology, Meharry Medical College, Nashville, TN 37208

²Departments of Pediatrics and Pharmacology & Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37212

Abstract

The focus of this study was to characterize the impact of gestational exposure to benzo(a)pyrene, [B(a)P] on modulation of glutamate receptor subunit expression that is critical for the maintenance of synaptic plasticity mechanisms during hippocampal or cortical development in offspring. Previous studies have demonstrated that hippocampal and/or cortical synaptic plasticity (as measured by long-term potentiation and S1-cortex spontaneous/evoked neuronal activity) and learning behavior (as measured by fixed-ratio performance operant testing) is significantly impaired in polycyclic aromatic or halogenated aromatic hydrocarbon-exposed offspring as compared to controls. These previous studies have also revealed that brain to body weight ratios are greater in exposed offspring relative to controls indicative of intrauterine growth retardation which has been shown to manifest as low birth weight in offspring. Recent epidemiological studies have identified an effect of prenatal exposure to airborne polycyclic aromatic hydrocarbons on neurodevelopment in the first 3 Years of life among inner-city children (Perera et al., 2006). The present study utilizes a well-characterized animal model to test the hypothesis that gestational exposure to B(a)P causes dysregulation of developmental ionotropic glutamate receptor subunit expression, namely the N-methyl-D-aspartate receptor (NMDAR) and α -amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptor (AMPA) both critical to the expression of synaptic plasticity mechanisms. To mechanistically ascertain the basis of B(a)P-induced plasticity perturbations, timed pregnant Long-Evans rats were exposed in an oral subacute exposure regimen to 0, 25 and 150 μ g/kg BW B(a)P on gestation days 14–17. The first sub-hypothesis tested whether gestational exposure to B(a)P would result in significant disposition in offspring. The second sub-hypothesis tested whether gestational exposure to B(a)P would result in downregulation of *early* developmental expression of NMDA and AMPA receptor subunits in the

*Corresponding author: Darryl B. Hood, Ph.D., Department of Neurobiology and Neurotoxicology, Center for Molecular and Behavioral Neuroscience, Meharry Medical College, Nashville, TN 37208, USA. Phone (615) 327-6358, FAX: (615) 327-6632. email: dhoo@mmc.edu.

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hippocampus of offspring as well as in primary neuronal cultures. The results of these studies revealed **significant**: 1) disposition to the hippocampus and cortex, 2) down-regulation of developmental glutamate receptor mRNA and protein subunit expression and 3) voltage-dependent decreases in the amplitude of inward currents at negative potentials in B(a)P-treated cortical neuronal membranes.

These results suggest that plasticity and behavioral deficits produced as a result of gestational B(a)P exposure are at least, in part, a result of down-regulation of *early* developmental glutamate receptor subunit expression and function at a time when excitatory synapses are being formed for the first time in the developing central nervous system. The results also predict that in B(a)P-exposed offspring with reduced *early* glutamate receptor subunit expression, a parallel deficit in behaviors that depend on normal hippocampal or cortical functioning will be observed and that these deficits will be present throughout life.

Introduction

Benzo(a)pyrene, is the prototypical polycyclic aromatic hydrocarbon (PAH) that is produced by the incomplete combustion of organic substances in processes such as trash incineration, coal burning, automobile exhausts, charcoal grilling, and in wood and paper processing processes (Ramesh et al., 2004; Dahlgren et al., 2003; Wormley et al., 2004; ASTDR, 1995). Human exposure to B(a)P can occur through the ingestion of contaminated food and water (Ramesh et al., 2004; Phillips, 1999) or via the inhalation of particulates in the ambient air (ATSDR, 1995; Hood et al., 2000).

Epidemiological studies have shown that unintended gestational exposure of the fetus to environmental contaminants, such as B(a)P, adversely affects fetal development, results in low birth weight and reduced head circumference that manifests as neurobehavioral deficits such as poorer outcome on selective aspects of cognitive and neuromotor functioning in offspring (Hack et al., 199; Perera et al., 2003; Landrigan et al., 2004). For example, in a sample of 263 nonsmoking inner-city African-American and Dominican women, the effects of gestational exposure to airborne PAHs on birth outcomes was monitored during pregnancy by personal air sampling in New York City. Among African Americans, high prenatal exposure to PAHs was associated with lower birth weight ($p = 0.003$) and smaller head circumference ($p = 0.01$) after adjusting for potential confounders. The study provides evidence that environmental contaminants at levels currently encountered in New York City adversely affect fetal development. (Perera et al., 2003)

A more recent prospective epidemiological study in support of the deleterious effects of gestational exposure to PAH's on cognitive functioning was reported recently by Perera et al., (2006). An effect of prenatal exposure to airborne polycyclic aromatic hydrocarbons on neurodevelopment in the first 3-years of life among inner-city children was identified. This prospective cohort study found that 3-year olds who had higher prenatal exposure to PAHs scored on average 5.69 points lower on cognitive tests than less-exposed children, even when controlling for other exposures and socioeconomic factors. The higher-exposed children also had twice the odds of developmental delay, suggesting an increased risk for performance deficits in language, reading, and math in the first years of school. The results in this article demonstrate for the first time that exposure to airborne polycyclic aromatic hydrocarbons *in utero* may affect cognitive development during childhood.

An important question that arises as a result of the studies discussed above is: Does gestational exposure to PAH's result in dysregulation of neuronal development at a time when synapses are being formed for the first time in the developing brain?. To date, there have been very few reports characterizing the toxic effects resulting from gestational PAH exposure with respect to development of the central nervous system (CNS). Exposure to B(a)P has been reported to

produce a variety of CNS effects including reductions in effects mediated by motor cortex (see Saunders et al., 2002) as well as reductions in long term potentiation and in hippocampal functioning in general (Wormley et. al. 2004 a+b). Significant reductions in behavioral learning in the resulting offspring (see Wormley et. al. 2004b) have also been reported and hypothesized to be mediated by dysregulation of developmental glutamate receptor subunit expression.

A long-term goal of this laboratory is to characterize the functional consequences of gestational exposure to PAH's which should lead to mechanistic explanations as to how developmental insult leads to long-term structural alterations and functional disturbances of the nervous system. With this in mind, we continue to utilize a susceptibility exposure paradigm in a timed-pregnant rat model as a means of determining the mechanisms of causality with respect to environmental contaminant brain burdens and the subsequent plasticity-related deficits associated with gestational exposure to B(a)P. A goal of the present study was to evaluate the cellular and molecular mechanisms associated with the functional consequences of dysregulation of early ionotropic glutamate receptor subunit expression as a result of gestational B(a)P exposure during the peak period of neurogenesis and synaptogenesis in the hippocampus. Glutamate receptors are highly involved in activity dependent synaptic plasticity and long-term changes in synaptic strength involved in hippocampal-and cortical based learning and memory consolidation. (Hood et al., 2006) Ionotropic glutamate receptors are pharmacologically profiled and are divided into three subclasses: N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainite receptors. Our findings reveal that, indeed, gestational exposure to B(a)P dysregulates temporal developmental mRNA and protein expression of certain glutamate receptor subunits in the offspring at a time when excitatory synapses are being formed for the first time in the developing brain. The results suggest a mechanism for the observed plasticity deficits subsequent to toxicant exposures reported in the literature (Gilbert, et al., 2003 and 2004, Hood et al., 2006) as well as for the subsequent toxicant-induced behavioral learning deficits associated with environmental toxicant exposure (Levin et al, 2001 and 2002; Wormley et al., 2004)

Methods and Materials

Power analysis was used to evaluate the proper numbers of offspring animals required from the standpoint of offspring litters as the statistical unit. From this analysis, six cohorts of six randomized timed-pregnant dams between three experimental groups were utilized: 1) a control group (vehicle exposed), 2) a 25 µg/kg body weight (BW) B(a)P exposed group and 3) a 150 µg/kg body weight (BW) B(a)P exposed group. In totality, 194 F1 generation pups were utilized consisting of sixty-four control, sixty-five 25 µg/kg body weight (BW) B(a)P exposed offspring and sixty five 150 µg/kg body weight (BW) B(a)P exposed offspring from thirty-six litters were used for the bioavailability and molecular biological studies described in this report. For each repetition, three sets of rats were required and was estimated that the variance between measures from litters would be 10% of the mean response. Therefore indicating that three successful experiments in each cohort would be required to detect 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-exposed offspring animals relative to the offspring control animals.

Animal Husbandry and Benzo(a)Pyrene Susceptibility Exposure Paradigm

All experiments were approved by the Institutional Animal Care and Use Committees of Vanderbilt University and Meharry Medical College and were performed according to Guidelines for Animal Experimentation as set forth by both institutions. Time-pregnant Long-Evans Hooded dams were obtained from Charles River Laboratories (Wilmington, MA) on

gestational day (GD) 10. Upon arrival, animals were housed individually in clear plastic cages with laboratory grade (heat-treated) pine shavings as bedding. Animals were quarantined for 2 days in the AALAC accredited Meharry Medical College animal care facility and were maintained in a controlled environment with a temperature at $21 \pm 2^{\circ}\text{C}$ and relative humidity of $50 \pm 10\%$ with a 12/12 hr light/dark cycle. Dams were fed commercial food (Rat Chow 5012; Purina Mills, St. Louis, MO). Water and food was available *ad libitum*.

Each dam was randomly assigned to an experimental group and on GD14-17, timed-pregnant dams were exposed, by oral gavage in a total volume of 0.875ml to 1) peanut oil or to 2) 50 $\mu\text{g/kg}$ B(a)P or 3) 150 $\mu\text{g/kg}$ BW B(a)P. From these exposures, ten litters resulted from each of the groups and two to three pups per litter per group per cohort (total of 3 cohorts) were removed on PND 2, 5, 10, 15, and 20 from litters and analyzed for B(a)P disposition and glutamate receptor subunit developmental expression. Primary neuronal cultures were generated “ex vivo” from GD18 timed-pregnant dams.

Brian Dissections

On PND 2, 5, 10, 15 and 20, offspring pups were randomly selected and removed from each litter, and euthanized by CO_2 exposure and were dissected for left and right cerebral cortex, hippocampus cerebellum, liver, and whole blood. On PND 2, whole brains were collected and pooled because of small size of pups. All samples were stored at -80°C , until ready for processing.

B(a)P Disposition Analysis from Hippocampus and Cerebral Cortex

Animals were euthanized by carbon dioxide asphyxiation. Brain regions (cerebral cortex and hippocampus), and livers were excised and collected at PND 2, 5, 10, 15, and 20. Tissues were transferred into cryovials and stored frozen at -70°C until analyzed. Because of the small volume of tissues, pooled samples (from a total of 5 pups/each PND) were used for analysis of B(a)P/metabolites. Two hundred and fifty to five hundred milligrams of tissue was weighed using a Cahn C-30 microbalance (Cerritos, CA) homogenized in two volumes of Tris-sucrose-EDTA buffer (0.25 M; pH 7.4). Ten microliters of sodium dodecyl sulfate was added to the mixture and vortexed for 1min. The homogenate was extracted with a solution containing methanol (5 ml), deionized water (1.5 ml) and chloroform (2 ml). Centrifugation was performed at $2000\times g$ for 20 min to facilitate separation of aqueous and organic phases. Subsequently, the organic phase was passed through anhydrous sodium sulfate to remove moisture. The organic phase was dried under N_2 , using a Meyer Analytical Evaporator (Organomation Associates Inc., Berlin, MA), resuspended in 0.5 ml of methanol and passed through Acrodisc filters (0.45 μm ; 25 mm diameter, Gelman Sciences, Ann Arbor, MI) to remove particulates. The final extracts were stored at 4°C in amber color screw top vials to prevent photodegradation until analyzed.

Sample analyses was conducted and is described in its entirety in Hood et al., (2000) by high performance liquid chromatography (Model 1050 Hewlett Packard, Wilmington, DE) equipped with a HP 1046 fluorescence detector. Briefly, using an automatic sampler, 30 μl samples were injected onto a C_{18} reverse phase column (ODS Hypersil, 5 μm , 250×4.6 mm; Hewlett Packard). The column (temperature 33°C) was eluted for 45 min at a flow-rate of 1.0 ml/min with a ternary gradient of water: methanol: ethanol (40:40:20) for 20 min, followed by the same gradient at a ratio of 30:46:24 for 10 min, 100% methanol for 10 min and then returning to the initial gradient for 5 min. The excitation and emission wavelengths for the detector were 244 and 410 nm, respectively. Identification and quantification of the metabolites were accomplished by comparing retention times and peak areas of samples with that of standards (National Cancer Institute Chemical Carcinogen Repository, Midwest Research Institute, Kansas City, MO), using HPLC 2 D Chemstation software (DOS series, Hewlett

Packard). The following B(a)P metabolite standards were used for identification and quantitation of metabolites: B(a)P-*trans*-4,5-dihydrodiol ($\pm$), B(a)P-*trans*-7,8-dihydrodiol ($\pm$), B(a)P-*trans*-9, 10-dihydrodiol ($\pm$), B(a)P-3,6-dione, 3-hydroxy benzo(a)pyrene and 9 – hydroxy benzo(a)pyrene.

mRNA Analysis

Total RNA was isolated from control and B(a)P-exposed offspring littermates. Cerebral cortex and/or SI barrel field cortex total RNA (PND240) was isolated with the QIAGEN (Valencia, CA) RNeasy Mini Kit. Briefly, approximately 30–50 mg of tissue was homogenized in Trizol Reagent (Invitrogen, Carlsbad, CA) using a 20G (0.9mm) needle attached to a sterile plastic syringe. Chloroform (0.2 volume) was added to homogenate and vortexed. The RNA samples were then centrifuged (13000 rpm, 15min, 4°C) and the supernatant were purified with QIAGEN (Valencia, CA). RNeasy mini spin column as specified in manufacturer's protocol. The purity of total RNA was determined by measuring the absorbance at 280nm and 260nm on a spectrophotometer; an absorbance ratio greater than 1.7 was deemed an acceptable measure of RNA purity. To minimize the contamination of genomic DNA, RNA samples were treated with QIAGEN (Valencia, CA) RNase-free DNase (1u/ μ g RNA, 15min). Total RNA samples were precipitated with 3M sodium acetate (pH 5.2) and 100% ethanol and stored at –80°C overnight. The precipitated RNA was then centrifuged (13000 rpm, 15min, 4°C), supernatant discarded and allowed to slightly air dry. The RNA pellet was redissolved in RNase-Free water to a concentration of 1 μ g/ μ l. RNA integrity was checked by visual examination of two sharp ribosomal RNA bands (28s and 18s) on a 1% agarose gel with 0.5 μ g/ml of ethidium bromide.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

To detect B(a)P induced temporal modulation of NR2A, NR2B, and GluR1 mRNA expression in F1 generation animals, semi-quantitative RT-PCR analysis was used. The cDNA synthesis and RT-PCR amplification were produced in one step using Promega's Access RT-PCR System. The sequences of designed primers were:

NR2A Forward: 5'-ATA CCG GCAGAA CTC CAC AC-3'

NR2A Reverse: 5'-CTC TTG CTG TCC TCC AGA CC-3'

The size of the amplified product is 441 bp.

NR2B Forward: 5'-AGA AAC CTG TCC TTC AGC GA-3'

NR2B Reverse: 5'-GTC AAC CAC CTC TGA CCG TT-3'

The size of the amplified product is 534 bp.

GluR1 Forward: 5'-CAA CAG CCT GTG GTT CTC CC-3'

GluR1 Reverse: 5'-CAT TGA TGG ATT GCT GTG GG-3'

The size of the amplified product is 791 bp.

The final concentrations of reagents in the PCR reaction system were as follows: 1X RT-PCR buffer, 1mM MgSO₄, 0.2mM of each deoxynucleosidetriphosphate (dNTP), 1 μ M of each primer, 0.1u/ μ l Reverse Transcriptase, 0.1u/ μ l DNA Polymerase, and 20ng of total RNA was used as template in each 50 μ l reaction volume. For quantitative analysis of gene expression, Ambion (Austin, TX) QuantumRNA™ 18sRNA Internal Standards Kit was used to amplify 18sRNA together with the specific target gene in one tube. The optimal ratio of 18sRNA primer: competitor for each gene was determined prior to RT-PCR so that two bands can be visualized together. RT-PCR cycling conditions were as follows: 48°C 45 minutes for reverse transcription, and then 94°C, 2 minutes for RT inactivation; 94°C 30 minutes→60°C 1

minute→68°C 2 minutes for PCR, there were 30–35 cycles. All PCR reactions were linear at the PCR cycling conditions given above. The PCR products were electrophoresed on 1.2% agarose gel with 0.5µg/ml ethidium bromide. The specificity of each band was confirmed by the size of the amplified products, corresponding, for example, to 534bp for NR2B, and 324bp for 18sRNA. The density of each band was measured by an AlphaImager™ 2000 Digital Imaging System (Alpha Innotech Corp; San Leandro, CA., using the protocol described in Hood et al., 2000). The levels of target gene mRNA expression are shown as the relative ratio between the intensity of the target-specific band and that of the 18sRNA band.

Statistical Analysis of mRNA Developmental Expression Profiles

Values are given as means ± S.E.M. in figures. A one-way analysis of variance (ANOVA) was used for the determination of statistical differences between control and offspring B(a)P exposed groups in NMDA NR2A, NR2B and GluR1 mRNA gene expression levels relative to 18sRNA. 18S was used as an internal standard for the RT-PCR analysis because this gene has been demonstrated to not be modulated as a result of B(a)P exposure and thus, not affect the expression of mRNA. 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 being normalized to the amount of rRNA it contains. rRNAs are also transcribed by a distinct polymerase from mRNAs, which may result in a different pattern of regulation of expression. 18S rRNAs have been found to be uniform in all rodent tissues tested including liver, brain, thymus, heart, lung, spleen, testes, ovary, kidney and embryo. Therefore, the choice of a housekeeping gene in toxicology studies is based on empirical determinations as to the effect of the toxicant on the system under study.

Since there was the expected within and between litter variation, mRNA expression levels were tested for statistical differences among different PNDs. All pairwise multiple comparisons were done by Student–Newman–Keuls method. The criterion for statistical significance was $p < 0.05$ in all cases.

Generation of Primary Neuronal Cultures

All experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University and were performed according to Guidelines for Animal Experimentation as set forth by Vanderbilt University. Rat cortical neuron cultures were prepared from E17 rat pups, as previously described (McLaughlin et al. 1998). Briefly, E17 Harlan Sprague-Dawley rat embryos were decapitated, and the brains rapidly removed and placed in a 35 mm petri-dishes with cold Hank's balanced salt solution (HBSS). The cortices were dissected under a dissection microscope and then were placed in another dish containing HBSS to further remove blood vessels and meninges from cortical tissues. The isolated cortices were then transferred to a petri-dish containing 0.6% (w/v) 0.22 mm filtered trypsin in HBSS for 30 minutes. After two washes in HBSS, the cortical tissues were mechanically dissociated with a glass Pasteur pipette. Dissociated cortical cells were plated on poly-L-ornithine-treated glass coverslips in 6-well plates, using a plating medium of glutamine-free DMEM-Eagle's salts (Invitrogen, Carlsbad, CA), supplemented with Ham's F12 (Hyclone, Logan, UT), heat-inactivated fetal bovine serum (Hyclone, Logan, UT), and penicillin/streptomycin (Sigma, St. Louis, MO), at a density of 700,000 cells per well. After 2 days *in vitro*, non-neuronal cell division was halted by a 1 day exposure to 10 µM cytosine arabinoside (Sigma, St. Louis, MO), and cultures were shifted to Neurobasal media (Invitrogen, Carlsbad, CA), supplemented with B27 (Invitrogen, Carlsbad, CA) and penicillin/streptomycin. Cells were maintained by changing the media every 2–3 days and grown at 37°C in a humidified atmosphere of 5% CO₂ in air.

Cells were treated three weeks after isolation with B(a)P, prepared as sterile solutions in treatment buffer, for 24 hr, at 37°C in a humidified atmosphere of 5% CO₂ in air. Treatment buffer consisted of MEM (Invitrogen, Carlsbad, CA) supplemented with 25 mM HEPES, 2× N2 media supplement (Invitrogen, Carlsbad, CA), 0.001% BSA (Sigma, St. Louis, MO). N-methyl-D-aspartate (NMDA, Sigma, St. Louis, MO) was used as a positive control for cytotoxicity at a final concentration of 100 mM in conjunction with 10 mM glycine.

Immunohistochemistry of Glutamate Receptor Subunit Proteins in Primary Neuronal Cultures

On day 9 following B(a)P exposure, coverslips were transferred to 6-well plates (right-side up, 1 slip/well) and fixed with 4% formaldehyde for ten minutes. Each well contained ~ 2.0 mL of 4% formaldehyde. Each coverslip was then washed with Dulbecco's Phosphate-Buffered Saline (1× PBS) three times. After fixation, neuronal cells were permeabilized with methanol that was prechilled at -20 degrees for 5 minutes. Primary neuronal cultures (on coverslips) were then incubated with the specified primary antibody (1:1000 in 10% FBS in PBS) for NR2B or GluR1 (rabbit, goat polyclonal antibodies; Santa Cruz Biotechnology, Santa Cruz, CA) in a 6-well plate RT for 1 hour. After washing with 1X PBS three times, neurons were then incubated with fluorescent, conjugated secondary antibody (either Alexa-Fluor 488 or 546; 1:100 in 10% FBS in PBS) for 1-hour RT. The coverslips were then washed face up in 1× PBS three times and once in distilled water. Coverslips were then mounted on cover slides using Thermo Immu-Mount glue and dried in the dark overnight at room temperature. The slides were mounted on an inverted Nikon TE2000 microscope. Images were collected using a 40×, 1.3 numerical aperture, oil immersion Plan Fluor objective lens and a side-mounted CoolSNAPHQ2 camera. Cells were chosen using DIC, and then fluorescence images were acquired using FITC and TRITC filter cubes. Nikon Elements software was used for automated image collection, and the fluorescence signal was captured for identical times in control and B(a)P-exposed cells.

Statistical Analysis of NR2B and GluR1 Protein Immunofluorescence in Control and B(a)P-Exposed Primary Neuronal Cultures

Fluorescence images were captured using an inverted Nikon TE2000E microscope. Images were collected using a 40×, 1.3 numerical aperture, oil immersion Plan Fluor objective lens and a side-mounted CoolSNAPHQ2 camera. Cells were chosen using DIC, and then fluorescence images were acquired using FITC HyQ and TRITC filter cubes. Nikon Elements software was used for automated image collection, and the fluorescence signal was captured for identical times in control and B(a)P-exposed cells in order to quantify any differences in the fluorescence intensity. A 223 × 164 μm section of a coverslip was analyzed for NR2B or GluR1 immunoreactive neurons. This process was repeated in five different sections of the coverslip to give an n=5 for each glutamate receptor subunit. The resulting values are given as means ± S.E.M. in Figure 7(inset). Statistical analyses between control NR2B and GluR1 protein immunofluorescence in primary neuronal cultures versus B(a)P-exposed NR2B and GluR1 protein immunofluorescence in primary neuronal cultures was conducted using the two-tailed, unpaired Student's t test Figure 7 (inset). The criterion for statistical significance was $p < 0.05$ in all cases.

Electrophysiology recordings

As pointed out in Cull-Candy and Leszkiewicz, (2004) initial biophysical characterizations are always prudent and necessary when working with primary neuronal cultures. As such, the data presented as Figure 8 is a characterization and validation study of the electrical properties of the neuronal culture as well as to find the measurements of greatest importance with regard to the effects of exposure to B(a)P. Primary neuronal cultures were exposed to various

concentrations of B(a)P (25 and 100nM). The neuronal recordings were defined as stable if there was less than 5% change in the resting membrane potential during the first 5 minutes of baseline recordings and if the seal resistance did not decrease to values below 1G Ω throughout the recordings. At high B(a)P concentrations (100nM) the baseline recordings were unstable, although we successfully obtained initial tight seals (1–5G Ω). Consideration was given to the possibility that in addition to the B(a)P-induced biological affects reported, B(a)P could possibly be reducing the membrane stability of the neurons in culture at high concentrations thereby decreasing the stability of the whole-cell patch clamp. Decreasing the B(a)P concentration to 25nM provided optimal conditions for electrophysiological characterization of the primary neuronal culture. Measurements revealed significant decreases in the amplitude of inward current in the B(a)P treated neurons as compared to control neurons and supports the conclusion of down-regulation of glutamatergic receptor subunit expression reported in Figure 7. The fact that a significant alteration in biophysical properties was quantifiable due to the ability to attain a stable patch-clamp at 25nM B(a)P and not at 100nM B(a)P further supports the dose sensitivity with regard to toxicant insult to temporal glutamatergic receptor subunit expression and function. Therefore, it is conceivable that these results would be more pronounced at higher concentrations of B(a)P (i.e. 100nM) if a stable patch-clamp was attainable.

Whole cell patch clamp recordings on control and 100nM B(a)P-exposed primary neuronal cultures were performed at room temperature using Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Whole cell currents were recorded using an Axopatch 200B with a low-pass Bessel filter set at 1kHz. Current-voltage relations were generated using a voltage step (1 sec) protocol ranging from –100 to 40 mV separated by 20 mV from a holding potential of –60 mV. Data were recorded and analyzed off-line using the software pCLAMP 10 from Axon Instrument as described previously (Khoshbouei, 2004). Patch electrodes were pulled from quartz pipettes on a P-2000 puller (Sutter Instruments, Novato, CA) with a tip resistance of 3–5 M Ω and filled with the pipette solution containing the following (in mM): 138 CsCl, 1.4 CaCl₂, 2 MgCl₂, 10 EGTA, 10 HEPES, 3 Na₂-ATP, and 0.2 Na₂-GTP, pH adjusted to 7.35 with CsOH and osmolarity adjusted to ~280 mOs/kg with sucrose. The bath solution contained in mM 146 NaCl, 5 KCl, 2.5 CaCl₂, 10 HEPES, 30 Dextrose, pH adjusted to 7.35 with NaOH and osmolarity adjusted to ~300mOs/kg with sucrose. Tight seals (1–5G) were obtained by applying negative pressure. The membrane was disrupted with additional suction and the whole cell configuration was obtained.

Results

There were no significant differences in birth indices between control dams and B(a)P-exposed dams and pups per litter were 10.0 ± 1.02 , 10.8 ± 1.8 , and 11.56 ± 0.65 , in the control, 25 $\mu\text{g/kg}$ B(a)P exposed, and 150 $\mu\text{g/kg}$ B(a)P exposed groups, respectively. During the gestational exposure period as well as the subsequent pre-weaning period there were no identifiable B(a)P-related effects on conventional/reproductive indices of toxicity. No convulsions, tremors, or abnormal movements were noted in any control dam or pup or in any B(a)P exposed dam or pup. There were no significant differences in the liver to body weight ratios of the B(a)P-exposed offspring as compared to control offspring. There were however, significant differences in the brain to body weight ratios between B(a)P-exposed offspring as compared to control offspring on certain postnatal days. Brain to body weight ratios for B(a)P-exposed offspring relative to control offspring were PND0=1.0, PND15=1.25 and PND30=1.33. It is quite likely that these increased brain to body weight ratios in B(a)P-exposed offspring resulted from B(a)P-induced intrauterine growth retardation (IGR).

B(a)P Metabolite Disposition

Because B(a)P is lipophilic, significant disposition to the brain is expected to occur and thus initiate potential neurotoxic mechanisms (Wu et. al. 2003, Hood et. al. 2001). Previous studies have reported a significant correlation between neurotoxic effects and metabolite concentrations in plasma and brain, supporting the contention that B(a)P metabolism plays an important role in modulating neurobiological effects (Wormley et al., 2004; Saunders et. al. 2002, Ramesh et. al., 2004). The time course of total B(a)P metabolite concentrations in cerebral cortex, hippocampus and liver in offspring pups is shown as Figure 1A and 1B. The data reveal an approximate 3-fold increase on PND 2 in total B(a)P metabolite disposition to cerebral cortex, hippocampus and liver as the gestational dosing regimen is increased from 25 µg/kg B(a)P to 150µg/kg B(a)P (Figure 1B) in pre-weaning offspring pups. No detectable levels of metabolites were found in the vehicle exposed control offspring. Further analysis of total metabolites in terms of percent distribution in offspring pre-weaning pup brains is shown in Figure 2 (A–C) for the 25µg/kgBW B(a)P exposed group and in Figure 3 (A–C) for the 150µg/kgBW B(a)P exposed group. These analyses resulted in the detection of metabolites of B(a)P. A dose-related increase and time-related decrease of the total metabolites concentration and a significant interaction effect of dose and time ($p < 0.005$) was also observed in the present study. In addition to the total B(a)P metabolite load per gram of tissue in brain regions, the qualitative distribution of metabolites in the aforementioned brain regions is also important as they determine the outcome of toxicity. The qualitative distribution of hippocampal and hepatic B(a)P metabolites is presented in Figure 2 and Figure 3. The metabolite distribution at exposure dose of 25 and 150µg/kgBW are given as a representative example.

The concentrations of B(a)P diol metabolites (4,5; 7,8 & 9,10 diols) were high up to PND 10, where as the hydroxy metabolites (3 & 9-OH) constituted higher percentages at PND 15 and 20. The differences between these two metabolite groups at each of the time points (PND) monitored were statistically significant ($P < 0.005$). The formation of diols during this early period of development is interesting in that the diols can be converted further into dihydrodiol epoxides. From a toxicity standpoint, the dihydrodiol epoxides are important as they are very reactive to nucleophilic attack by nucleophilic sites in cellular macromolecules. The predominance of hydroxy metabolites at PND 15 and 20 indicate that the mechanism of detoxification may be more prominent at later stages of development. The distribution profile of B(a)P metabolites among the hepatic and brain tissues were similar, which is consistent with the oral route of exposure where the metabolic processing of B(a)P by liver is reflected in the distribution of metabolites in extrahepatic tissues. It is readily apparent from analysis of the time course disposition profiles at both doses that the percent distribution of the 7,8,-dihydrodiol metabolite concentrates in the hippocampus during the pre-weaning testing period. The quantitative profile of B(a)P metabolites is also consistent with the oral gavage route of exposure utilized in the present study as opposed to the same generated as a result of inhalation of B(a)P aerosol that we previously reported in Wu et al., (2003).

Metabolism of B(a)P in primary neuronal cultures and whole brain tissues does differ markedly. The same cytochrome P450 (CYP) enzymes and glucuronosyltransferases that are expressed in neuronal cells isolated from the developing rat brain (Martinasevic et al., 1998, Gilbert et al., 2003) are also expressed in different cell types such as glial (Geng and Strobel, 1997), and astrocytes (Nicholson and Renton, 1999), and also whole brain tissues (Strobel et al., 1997). Additionally, Strobel et al. (1997) have shown that the content and activity of CYP in glioma cells is similar to that observed in whole brain tissues. Thus, it can be safely extrapolated that the metabolite profile generated as a result of biotransformation in *in vitro* primary neuronal cultures parallels that of *in vivo* exposure. Given the fact that measurable levels of B(a)P metabolites are detected subsequent to acute exposure, it is very likely that the continuous buildup of the metabolites as a result of chronic exposure would result in neuronal toxicity.

B(a)P-induced Suppression of Temporal Developmental Expression of Glutamate Receptor Subunits

As a means of testing whether gestational B(a)P exposure would result in modulation of early hippocampal developmental expression of NMDA and AMPA receptor subunits in offspring, semi-quantitative PCR was employed. Previous data (Wormley et al., 2004; Hood et al., 2006) indicated that late glutamate receptor subunit expression was modulated subsequent to B(a)P aerosol exposure. This previously reported data suggested that gestational B(a)P-exposure has the potential to reduce both hippocampal and cortical synaptic plasticity activity via alterations in glutamate receptor subunit expression thereby affecting NMDA and AMPA receptor activity. Therefore, glutamate receptor subunits (NR2A, NR2B, and GluR1) were evaluated on PND 2, 5, 10, 15, and 20 for *early* developmental expression.

As reviewed by Cull-Candy et al., (2004), NR2A expression is reduced at birth and increases with time. NR2B expression is abundant at birth and decreases with time. Therefore, NR1/NR2B to NR1/NR2A expression ratio is inversely proportional to time and this can be used as a starting point to assess the effects of gestational B(a)P exposure on the developmental mRNA expression of these glutamate receptor subunits.

Analysis of hippocampal mRNA developmental expression for NR2A normalized to 18sRNA expression in control and B(a)P-exposed offspring is shown as Figure 4. The developmental expression profile for control offspring are consistent with what is known in the literature. NR2A temporal developmental mRNA expression was found to gradually increase over the pre-weaning sampling period in control offspring. Conversely, in 150µg/kg B(a)P-exposed offspring, NR2A temporal developmental expression was found to be dysregulated as a result of B(a)P exposure. A significant 20% reduction in NR2A mRNA developmental expression is apparent on PND2 and by PND20 is reduced by approximately 25% in B(a)P-exposed offspring as compared to controls. The overall B(a)P-induced dysregulation of temporal developmental expression and the significant reductions in the developmental mRNA expression of the NR2A subunit are less robust in the 25mg/kg B(a)P-exposed offspring but are nevertheless present at later preweaning postnatal days (PND15 and PND20).

The hippocampal mRNA developmental expression for NR2B normalized to 18sRNA expression in control and B(a)P-exposed offspring is shown as Figure 5. Once again, the developmental expression profile for control offspring is consistent with what is known in the literature. In general, NR2B temporal developmental mRNA expression gradually declines with postnatal age (Cull-Candy et al., 2004). In control offspring, NR2B mRNA developmental expression shows a general trend for gradually declining over the pre-weaning period tested. Conversely, in 150µg/kg BW B(a)P-exposed offspring, a significant 75% precipitous reduction in NR2B developmental mRNA expression occurs from PND2 to PND20. Significant reductions in the temporal mRNA developmental expression of the NR2B subunit are once again, less robust in 25µg/kg BW B(a)P-exposed offspring but are nevertheless significantly reduced by approximately 35% by PND20.

Interestingly and consistent with the recent results reported for gestational TCDD-exposure effects on single cell responses in Hood et al., (2006), the AMPA component as measured by GluR1 temporal mRNA expression normalized to 18sRNA expression revealed a robust, significant 50% down-regulation in the 150µg/kg BW B(a)P-exposed offspring on by PND20 as compared to offspring controls (Figure 6). Overall, the results from studies quantifying early postnatal glutamate receptor subunit mRNA developmental expression revealed a dose-dependent down-regulation of NR2A, NR2B, and GluR1 expression as a result of gestational B(a)P exposure. High-level B(a)P exposure (150µg/kg BW) consistently and reproducibly resulted in a robust, significant reduction in NR2A, NR2B and GluR1 mRNA expression. Early postnatal down regulation of glutamate receptor subunit expression with low-level (25 µg/kg

BW) B(a)P exposure was not as robust as high level exposure but did consistently cause statistically significant reductions in expression as compared to control offspring. The results from these quantitative assays suggest a dose-dependent dysregulation of *temporal* developmental expression of glutamate receptor subunits as a result of gestational B(a)P exposure.

To further examine our hypothesis, simultaneous determinations of the effect of B(a)P exposure on neuronal glutamate receptor subunit expression was conducted utilizing *ex vivo* primary neuronal cultures (see methods and materials section). Primary neuronal cultures were exposed to 100nM B(a)P in the growth medium for 24 hours and the immunofluorescence for NR2B and GluR1 protein expression was visualized by fluorescence microscopy. The results from control and 100nM B(a)P-exposed *ex vivo* primary neuronal cultures is shown as a panel in Figure 7 (panels A–H). Consistent with the molecular biology studies, a robust down-regulation of glutamate receptor subunit protein expression (both GluR1 and NR2B) can be clearly observed as a result of exposure (Figure 7; panels F–H) of the primary neuronal culture to 100nM B(a)P as compared to the control culture (Figure 7; panels B–D). The immunofluorescence for the control primary neuronal culture for NR2B and GluR1 is shown in panels B and C in Figure 7. The merged image in panel D serves as a point of reference and represents the total co-localization expression of NR2B and GluR1 on the neuronal membrane. Conversely, panels F and G in Figure 7 show that NR2B and GluR1 fluorescence was consistently suppressed following exposure of the primary neuronal culture to 100nM B(a)P. The merged image in panel H serves represents the total co-localization expression of NR2B and GluR1 on the neuronal membrane from exposed neurons and is significantly reduced by greater than 60%. Double blind visual observations did not reveal a morphological difference between control and B(a)P exposed (100nM) *ex vivo* neuronal cell cultures. Differential Interference Contrast (DIC) microscopy was utilized to determine if morphological changes occurred as a result of B(a)P-exposure to primary neuronal cultures (panel E) as compared to control *ex vivo* neuronal cultures (panel A) and revealed no apparent significant differences.

Finally, in order to validate the results from the “*ex vivo*” primary neuronal cultures, cortical neurons were voltage clamped using whole cell configuration of the patch clamp technique and held at -60mV , in a nominally Mg^{2+} free external solution (see materials and methods). The current-voltage (I–V) relationship of control and 25nM B(a)P-exposed cortical neurons was nearly linear between -80 and $+20\text{ mV}$ (Fig 8A). Although there were no apparent differences between control and B(a)P-exposed cortical neurons at positive potentials there was a robust voltage-dependent decrease in the inward currents recorded at negative potentials as shown in Fig 8B ($t = -2.92789$, $p < 0.0429$). There were no differences in the average reversal potentials (E_{rev}) for control and 25nM B(a)P-exposed cortical neurons ($1.24\text{ mV} \pm 1.02$, and $1.6\text{ mV} \pm 2.7$, for control and B(a)P-exposed cortical neurons respectively), values not significantly different from zero and close to the equilibrium potentials for non-specific cation channel/s calculated from the given extra- and intracellular concentration. Figure 8C represents the quantitative plot showing the robust voltage-dependent decrease in the inward current recorded at negative potentials in the 25nM B(a)P-exposed cortical neurons as compared to control. Although these results are straightforward, gestational B(a)P exposure might instigate alteration of neuronal plasticity mechanisms causing deficits in long term potentiation (LTP) and/or long term depression (LTD) in various brain regions. In fact, previously, we have reported a decrease in the amplitude of hippocampal LTP subsequent to gestational exposure to B(a)P (Wormley et al., 2004). The present study adds new information to the field by illuminating a possible underlying molecular mechanism that is involved in B(a)P-mediated decreases in LTP upon acute exposure (in primary neuronal culture) as well as subsequent to gestational exposure. We have used complementary molecular, biochemical and biophysical approaches to quantify an apparent B(a)P-mediated alteration in ionotropic glutamate receptor

subunit expression. Our previously published data and current results set the stage for future characterizations of B(a)P-induced alterations in neuronal plasticity.

Although the exact underlying mechanism/s for B(a)P-mediated toxicity is not fully understood, both B(a)P and its metabolites are toxic (for review see Knafla et al., 2006; Wormley and Hood, 2004; Hood et al. 2006). *In vitro* studies with human endothelial cells (RF24 cell line and primary HUVEC), indicated that acute exposure to B(a)P causes a dose- and time-dependent cell toxicity (Knaapen AM, 2007). Furthermore, Chaudhary et al., recently reported production of reactive oxygen species in both 22Rv1 and PrEC cells upon exposure to acute B(a)P leading to cell toxicity (Chaudhary et al., 2007). Thus, B(a)P-mediated toxicity might be due to both its own oxidative properties, its reactive metabolites or both. Although it is not known whether B(a)P-mediated toxicity is due to its direct toxicity, the toxicity of its metabolites or both, our results suggest that both gestational and acute exposure to B(a)P produce comparable toxicity providing a new experimental model for future studies evaluating B(a)P-mediated neurotoxicity.

Discussion

The principal interpretation of the experiments described in this report within the context of previously reported studies (Gilbert, 2003; Gilbert, 2004; Hood et al, 2006; Wormley et al, 2004 a + b) is that gestational B(a)P exposure reduces early developmental glutamate receptor subunit expression in offspring at a time when excitatory synapse are being formed for the first time in the CNS. Recently, Grova et al., 2007 demonstrated that chronic exposure to B(a)P in adult mice modulates gene expression of the NMDA NR1 subunit in brain areas that are highly involved in cognitive function like the hippocampus. The results from this novel study suggest a relationship between the expression of functional NMDA-R1 mRNA and impairment of short-term and spatial memory as a function of B(a)P exposure levels.

The reductions in hippocampal (or cortical see- Hood et al., 2006) glutamate receptor subunit expression in the present study can be correlated and are most likely causative to the previously reported physiological deficits as measured by LTP in the dentate gyrus of B(a)P or TCDD-exposed offspring and both spontaneous/evoked cortical activity of TCDD-exposed offspring. Normal functioning of the hippocampus and/or S1 cortex was found to be, at least in part, impaired for at least one hundred postnatal days in offspring after gestational B(a)P-exposure as measured by decreases in behavioral learning (Wormley et al., 2004). Thus, within the context of the present study, the previously reported physiological deficits (Hood et al, 2006; Wormley et al, 2004) are consistent with gestational B(a)P and TCDD-induced suppression of NMDA and AMPA-mediated single cell responses in offspring.

Gestational B(a)P-exposure effects were shown by Wormley et al. (2004) to induce a 50% inhibition in the induction of long-term potentiation (LTP) in the hippocampus. LTP is defined as a persistent, activity-dependent increase in the strength of synaptic transmission induced usually induced by high frequency stimulation of excitatory inputs to hippocampal cells. This potentiated effect persists for hours to weeks and requires postsynaptic depolarization sufficient to recruit NMDA receptor activation and subsequent Ca⁺⁺ influx into the cell through receptor-activated voltage-dependent channels (Collingridge, 1987). When the offspring from timed pregnant dams that were administered B(a)P (100µg/m³) by inhalation on gestational day 14–17 were tested following a brief volley of conditioning stimuli (100 Hz, 1 s, three times at 5 s intervals), very robust LTP was found in control rats. However, under the same conditions LTP was reduced by 50% in B(a)P-exposed offspring (Wormley et al., 2004). Based on the present study, NMDA as well as AMPA receptors are most likely crucial for this type of plasticity also. The reduction in LTP in B(a)P-exposed offspring and spontaneous/evoked activity in

TCDD-exposed offspring is highly likely to be due to down-regulation of glutamate receptors in general.

The results of the present study also corroborate the findings that gestational B(a)P-exposure induced deficits in behavioral learning in offspring correlate with early alterations in NMDA subunit expression. The developmental mRNA expression of the NR1, NR2A, and NR2B subunits in male littermates on PND 60, 65, and 70 was evaluated and reported in Wormley et al., (2004 a+b) and Nayyar et al. (2003). The obligatory NR1 mRNA is not significantly altered on PND 60, 65, nor 70 by gestational exposure to 100 μ g/m³ B(a)P aerosol. In the same brains, NR2A mRNA expression was significantly up-regulated, and NR2B mRNA expression was significantly down-regulated as compared to controls. Recently, Hood et al., (2006) reported similar findings in offspring TCDD-exposed brains in which pregnant dams were exposed to TCDD (700 ng/kg) by gavage. Gestational TCDD-exposure was found to downregulate early mRNA expression of the NR2B subunit as well as the GluR1 subunit in the cerebral cortex during the pre-weaning period.

Our data are in agreement with other recent findings (Grova et al., 2007; Kakeyama et al. (2001) and suggest that gestational B(a)P-exposure has selective effects on the temporal early developmental expression of NMDA and AMPA receptor subunits. Subunit down-regulation may lead to long-term decreases in synaptic functioning. The fact that the fetus was exposed to B(a)P during development supports the conclusion that regulation is impaired that normally results in expected NMDA and AMPA receptor profiles, and dysregulation of these profiles are very likely to be important factors in the observed B(a)P-induced decreases in neuronal activity (LTP).

The neurotoxicity induced by gestational exposure to B(a)P may alter hippocampal or cortical axonal properties, making them less able to support high frequency conduction of action potentials. Based on the molecular and electrophysiological studies reported here, there now exists a point of reference for the residual levels of B(a)P metabolites that give rise to alterations in early developmental glutamate receptor subunit composition and thus altered neuronal membrane potential in offspring brain that will serve as a basis to address how axonal conduction relates to changes in hippocampal or cortical responsiveness. In this context, it has been reported that some PAHs and their metabolites modulate cAMP-dependent ion secretion in various cell types which has a dramatic impact on the membrane potential in these various cell types. For example, PAHs such as B(a)P, 7,12-dimethylbenz(a)anthracene (DMBA), dibenz(a,h)anthracene, and 9,10-dimethylanthracene have been shown to cause a sustained elevation of intracellular calcium [Ca²⁺] in T-lymphocyte cells (Krieger et al., 1994) which was ascribed to inhibition of sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (Krieger et al., 1995). Similarly, the diol-epoxide metabolites of B(a)P have been found to increase the cytosolic Ca²⁺ in airway epithelial cells (Jyonouchi et al., 2001). Also, Ito et al. (2004) reported that the PAH compound fluoranthene potentiates cAMP-dependent anion secretion in airway epithelial cells, thus regulating the membrane potential of these cells.

Once the blood brain barrier is crossed, enhanced disposition of B(a)P metabolites along neurons has been shown to be facilitated by axonal transport mechanisms (Persson et al., 2002). Additionally, It has also been reported that the PAH compound DMBA affects the organization of neuronal plasma membrane in hypothalamus (Garcia-Segura et al. 1992) which reinforces and supports our contention that the disposition of B(a)P metabolites to critical structures in the developing CNS is, in part, causative to the neurotoxicity observed on offspring as a result of gestational exposure. Gestational B(a)P-exposure may have differential effects on the specialized subunits of glutamate receptor. Down-regulation of this receptor may lead to a sustained decrease in synaptic functioning. If gestational exposure modulates

glutamate receptor mRNA temporal developmental expression, then this may contribute to the reduced capacity for behavioral learning subsequent to gestational B(a)P exposure.

The findings from the present study are in agreement with other reports in the literature that document similar effects of other polycyclic aromatic hydrocarbons on learning and memory mechanisms. For example, Konstandi et al. (1997) reported that repeated treatment of rats with 3-methylcholanthrene at 25 mg/kg, i.p., two times per week, for 1 month resulted in a reduction of learning ability in a two-way active avoidance procedure. Other studies have reported similar findings whereby deficits in learning and memory were induced subsequent to gestational toxicant-exposure (Grova et al., 2006; Wormley et al., 2004; Widholm et al., 2003; Gilbert et al., 2000). The conclusions drawn from the present study, however, stand in stark contrast to the conclusions drawn from the Grova et al., (2006) and may be attributable to the use of gestational susceptibility exposure paradigm (low-dose = 150µg/kg BW) as opposed to a conventional sub-acute exposure paradigm (high-dose = 200mg/kg BW).

The NR1 subunit is required for the formation of functional NMDA receptor channel (Cull-Candy and Leskiewicz, 2004). Activation of the NMDA and AMPA subtypes of glutamate receptors are required for the modulation of learning and memory mechanisms and synaptic plasticity processes in the hippocampus and somatosensory cortex (Hood et al., 2006). The previously mentioned study from Wormley et al., 2004 failed to demonstrate an effect of exposure on modulation (up or down) of the obligatory NR1 subunit expression during the period of testing for long term potentiation from offspring hippocampus. These findings are in contrast, the findings from the Grova et al., (2006) study which showed that sub-acute B(a)P exposure significantly upregulates NR1 subunit receptor levels in the hippocampus of adult mice. Nevertheless, the conclusion from Grova et al., (2006) was that B(a)P exposure (depending on the dose and the brain region) influences the regulation of the glutamatergic neurotransmission.

Finally, gestational B(a)P-exposure, in particular, and toxicant exposure, in general, has been shown to have differential effects on specialized subunits of glutamate receptors. (Grova et al., 2006; Hood et al., 2006; Chen et al., 2004; Wormley et al., 2004a; Wormley et al., 2004b; Guilarte and McGlothlan, 1998 and Nihei and Guilarte, 1999). Down-regulation of this receptor leads to a sustained decrease in synaptic functioning at a time when excitatory synapses are formed for the first time. If gestational exposure to B(a)P modulates temporal glutamate receptor subunit mRNA developmental expression then this may contribute to the reduced capacity for the homeostatic maintenance of synaptic plasticity mechanisms during development and thus, may contribute to subsequent behavioral learning deficits.

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A **B**

Figure 1. Time-Course Distribution of Bioavailable B(a)P Metabolites in Cerebral Cortex, Hippocampus and Liver of Offspring Pups
Timed-pregnant dams received in A) 25 or B) 150 μ g B(a)P/kg BW via gavage on GD14-17. Offspring pups were sacrificed on PND 2, 5, 10, 15, and 20 and metabolite levels were determined as outlined in the methods and materials section. Values represent mean + S.E.M.

Figure 2. Percentage distribution of B(a)P metabolites in Offspring Pups

A) cerebral cortex, B) hippocampus, and C) liver. Timed pregnant dams were dosed with 25 μ g B(a)P/kg BW. Offspring pups were sacrificed on PND 2, 5, 10, 15, and 20 and metabolite concentrations were determined and authenticated using standards obtained from the NCI as outlined in the methods and materials section.

A **B** **C**

Figure 3. Percentage distribution of B(a)P metabolites in Offspring Pups

A) cerebral cortex, B) hippocampus, and C) liver. Timed pregnant dams were dosed with 150 μ g B(a)P/kg BW. Offspring pups were sacrificed on PND 2, 5, 10, 15, and 20 and metabolite concentrations were determined and authenticated using standards obtained from the NCI as outlined in the materials and methods section.

Figure 4. Offspring Pre-weaning Hippocampal Developmental Expression Profile of NR2A mRNA on PND 2, 5, 10, 15 and 20 following gestational exposure to B(a)P on GD14–17

Panel A shows the electrophoretic agarose gel results from semi-quantitative RT-PCR. The upper band is the NR2A subunit (441 bp) and the lower band is the internal control, 18sRNA (324bp). Panel B displays a histogram of the densitometric quantitation of the relative expression of target to internal 18sRNA control. M represents the DNA marker. Lanes 1–3 Offspring NR2A mRNA levels for PND2; (lane 1) white bar-control, (lane 2) black bar-50µg/kg B(a)P, (lane 3) grey bar-150µg/kg B(a)P. Lanes 4–6 Offspring NR2A mRNA levels for PND5; (lane 4) white bar-control, (lane 5) black bar-50µg/kg B(a)P, (lane 6) grey bar-150µg/kg B(a)P. Lanes 7–9 Offspring NR2A mRNA levels for PND10; (lane 7) white bar-control, (lane 8) black bar-50µg/kg B(a)P, (lane 9) grey bar-150µg/kg B(a)P. Lanes 10–12 Offspring NR2A mRNA levels for PND15; (lane 10) white bar-control, (lane 11) black bar-50µg/kg B(a)P, (lane 12) grey bar-150µg/kg B(a)P. Lanes 13–15 Offspring NR2A mRNA levels for PND20; (lane 13) white bar-control, (lane 14) black bar-50µg/kg B(a)P, (lane 15) grey bar-150µg/kg B(a)P. *p<0.05 relative to control on the respective PND.

Figure 5. Offspring Pre-weaning Hippocampal Developmental Expression Profile of NR2B mRNA on PND 2, 5, 10, 15 and 20 following gestational exposure to B(a)P on GD14–17

Panel A shows the electrophoretic agarose gel results from semi-quantitative RT-PCR. The upper band is the NR2B (534 bp) subunit and the lower band is the internal control, 18sRNA (324bp). Panel B displays a histogram of the densitometric quantitation of the relative expression of target to internal 18sRNA control. M represents the DNA marker. Lanes 1–3 Offspring NR2B mRNA levels for PND2; (lane 1) white bar-control, (lane 2) black bar-50µg/kg B(a)P, (lane 3) grey bar-150µg/kg B(a)P. Lanes 4–6 Offspring NR2B mRNA levels for PND5; (lane 4) white bar-control, (lane 5) black bar-50µg/kg B(a)P, (lane 6) grey bar-150µg/kg B(a)P. Lanes 7–9 Offspring NR2B mRNA levels for PND10; (lane 7) white bar-control, (lane 8) black bar-50µg/kg B(a)P, (lane 9) grey bar-150µg/kg B(a)P. Lanes 10–12 Offspring NR2B mRNA levels for PND15; (lane 10) white bar-control, (lane 11) black bar-50µg/kg B(a)P, (lane 12) grey bar-150µg/kg B(a)P. Lanes 13–15 Offspring NR2B mRNA levels for PND20; (lane 13) white bar-control, (lane 14) black bar-50µg/kg B(a)P, (lane 15) grey bar-150µg/kg B(a)P. * $p < 0.05$ relative to control on the respective PND.

Figure 6. Offspring Pre-weaning Hippocampal Developmental Expression Profile of GluR1 mRNA on PND 2, 5, 10, 15 and 20 following gestational exposure to B(a)P on GD14–17

Panel A shows the electrophoretic agarose gel results from semi-quantitative RT-PCR. The upper band is the GluR1 (791 bp) subunit and the lower band is the internal control, 18sRNA (324bp). Panel B displays a histogram of the densitometric quantitation of the relative expression of target to internal 18sRNA control. M represents the DNA marker. Lanes 1–3 Offspring GluR1 mRNA levels for PND2; (lane 1) white bar-control, (lane 2) black bar-50µg/kg B(a)P, (lane 3) grey bar-150µg/kg B(a)P. Lanes 4–6 Offspring GluR1 mRNA levels for PND5; (lane 4) white bar-control, (lane 5) black bar-50µg/kg B(a)P, (lane 6) grey bar-150µg/kg B(a)P. Lanes 7–9 Offspring GluR1 mRNA levels for PND10; (lane 7) white bar-control, (lane 8) black bar-50µg/kg B(a)P, (lane 9) grey bar-150µg/kg B(a)P. Lanes 10–12 Offspring GluR1 mRNA levels for PND15; (lane 10) white bar-control, (lane 11) black bar-50µg/kg B(a)P, (lane 12) grey bar-150µg/kg B(a)P. Lanes 13–15 Offspring GluR1 mRNA levels for PND20; (lane 13) white bar-control, (lane 14) black bar-50µg/kg B(a)P, (lane 15) grey bar-150µg/kg B(a)P. * $p < 0.05$ relative to control on the respective PND.

Figure 7. NMDA-NR2B and AMPA-GluR1 Subunit Expression is Down-Regulated in *ex vivo* Primary Cortical Neuronal Cultures following exposure to 100nM B(a)P

Panels A–D; Control Cortical Neuronal Cultures. Panel A; DIC microscopy image of Control Cortical Neurons. Panel B; GluR1 (red) protein expression on control cortical neuronal membrane. Panel C; NR2B (green) protein expression on control cortical neuronal membrane. Panel D; Co-localization of GluR1 and NR2B (yellow) protein expression on cortical neuronal membrane. Panels E–H; 100nM B(a)P-exposed Cortical Neuronal Cultures. Panel E; DIC microscopy image of 100nM B(a)P-exposed Cortical Neurons. Panel F; GluR1 (red) protein expression after 100nM B(a)P-exposed cortical neuronal membrane. Panel G; NR2B protein expression in 100nM B(a)P-exposed Cortical Neurons. Panel H; Merged GluR1 and NR2B

protein expression in 100nM B(a)P-exposed Cortical Neurons. Lower Panel: Analysis of NR2B and GluR1 Protein Immunofluorescence in Control and B(a)P-Exposed Primary Neuronal Cultures. A 223×164 um section of a coverslip was analyzed for NR2B or GluR1 immunoreactive neurons. This process was repeated in five different sections of the coverslip to give an n=5 for each glutamate receptor subunit. The resulting values were represented as means $\pm$ S.E.M. Statistical analyses between control NR2B and GluR1 protein immunofluorescence in primary neuronal cultures versus B(a)P-exposed NR2B and GluR1 protein immunofluorescence in primary neuronal cultures was conducted using the two-tailed, unpaired Student's t test *The criterion for statistical significance was $p < 0.05$.

Figure 8. B(a)P-exposure (25nM) Induces a Voltage-Dependent Decrease in the Inward Currents of Cortical Neurons

Cortical neurons were voltage clamped using whole cell configuration, in a nominally Mg^{2+} free external solution. Current-voltage relations were generated using a voltage step (1 sec) protocol ranging from -80 to 20 mV separated by 20 mV from a holding potential of -60 mV. (A) Representative I-V currents for untreated and 25 nM B(a)P-exposed cortical neurons. There is a robust voltage-dependent decrease in the magnitude of inward currents at negative potentials in the 25 nM B(a)P-exposed cortical neurons. (B) Representative current traces obtained from control and 25 nM B(a)P-exposed cortical neurons at -80 mV using the same experimental configuration as in (A). (C) Bar graph shows pA inward current recorded at -80 mV in control and 25 nM B(a)P-exposed cortical neurons ($t = -2.92789$, $p < 0.0429$, $n=3-5$).