PERINATAL EXPOSURE TO AROCLOR 1254 INDUCES LONG-TERM SUPPRESSION OF OSMOTICALLY INDUCED NITRIC OXIDE SYNTHASE ACTIVITY IN NEUROENDOCRINE CELLS OF THE RAT SUPRAOPTIC NUCLEUS

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Abstract
Magnocellular neuroendocrine cells (MNCs) within the supraoptic nucleus (SON) release vasopressin (VP) from both axon terminals in the posterior pituitary and centrally from soma/dendrites. In response to hyperosmotic stimulation, circulating VP released from this hypothalamo-neurohypophysial system (HNS) acts as a hormone to promote anti-diuresis and cardiovascular control whereas central release serves an autoregulatory role governing hormone output of VP. Previous work in our laboratory demonstrates that polychlorinated biphenyls (PCBs) markedly inhibit central VP release from SON punches in response to physiological activation. Because dehydration-induced central VP release has been shown to be dependent on nitric oxide (NO), disruption of NO signaling may be one mechanism through which PCBs act on the MNC system. To test this we used NADPH-d histochemistry to measure NO synthase (NOS) activity in SON of 15 month-old male rats perinatally exposed to Aroclor 1254 (30mg/kg/d; GD 10-19). Our findings indicate that PCBs compromise the upregulated activity of NOS in SON MNCs triggered by hyperosmotic challenge suggesting that developmental Aroclor 1254 exposure induces long-term effects on NOS activity within the HNS. Because central VP also promotes cognitive/executive functions and neurotrophism these findings could apply broadly to higher brain function and neurodevelopment. Support: NSF, UCMEXUS (MCC) and UCTSRTP Lead (CC).

Introduction
Persistent organic chemicals used in industrial applications are of major concern for environmental contamination and human health. Chronic exposure to polychlorinated biphenyls (PCBs) are persistent organohalogen compounds can lead to endocrine disruption and neurotoxicity[1]. Although the manufacture of PCBs, including the industrial mixture, Aroclor 1254, was discontinued in 1977; they are still found in significant quantities in the environment and in human tissues such as breast milk[2,3]. A number of studies suggest that Aroclor 1254 has the potential to disrupt endocrine homeostasis such as by decreasing serum thyroxin, estradiol and testosterone[4,5].

Disrupted intracellular signaling is a potential mechanism by which chemicals might alter nervous system function. Thus, brain function, including cognition, may be affected by neurotoxicants through changes in intracellular signaling at critical phases during development[6]. Although the mechanisms subserving the intracellular actions of PCBs remain to be fully elucidated, there are several well-documented modes of action associated with PCB-induced neurotoxicity including 1) altered intracellular Ca^{2+} homeostasis and modulation of PKC, 2) altered thyroid hormone levels and 3) changes in neurotransmitter systems[1]. Increasing evidence suggests that Nitric oxide (NO) signaling may also be disrupted by PCBs. NO which is produced by NO synthase (NOS), is a gaseous neurotransmitter. NO serves is implicated in LTP, learning and memory processes, neuroendocrine function and neurodegeneration[7-10]. Previously, it has been demonstrated that di-ortho-substituted PCB congeners inhibit both cytosolic and membrane NOS *in vitro*[11]. However, work by Canzoniero and colleagues (2006) using cultured neuroblastoma cells suggests that high doses of Aroclor 1254 may augnemt intracellular NO leading to cell death[12].

Magnocellular neuroendocrine cells (MNCs) within the supraoptic nucleus (SON) of the hypothalamus release vasopressin (VP) from both axon terminals in the posterior pituitary and locally from soma/dendrites. In response to dehydration, circulating VP acts as an anti-diuretic hormone to promote water retention, whereas central release may autoregulate MNCs and regulate systemic output of VP[13]. In addition, centrally released VP participates in cognitive/executive functions such as learning, memory and social behavior[14]. Since physiological activation of MNCs enhances both neuronal NOS gene and protein expression in SON, and NO signaling is a critical mediator of

stimulated central VP release, nitric oxide (NO) has been described as an activity-dependent modulator of the magnocellular neurosecretory system[15-18]. Previous work in our laboratory demonstrates that PCBs and PBDEs significantly reduce physiologically-activated central VP release from SON punches[19,20]. Moreover, certain PCB congeners have been shown to inhibit NOS activity *in vitro*[11]. In combination, these data suggest that PCBs may compromise NO-dependent central VP release by modulating NOS activity *in vivo*. We used nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry to estimate NOS function in paraformaldehyde-fixed tissue[21,22]. The rise in NADPH diaphorase staining is reported to coincide with a respective rise in the amount of NOS protein, NOS mRNA and/or NOS enzyme; therefore, this method can be used as an indicator of increased NOS function[23-25]. NOS activity was measured in fixed SON sections of 15 month old male rats exposed *in utero* (30mg/kg/d; GD 10-19) to Aroclor 1254. Our data indicate that early developmental PCB exposure significantly compromises the promotion of NOS activity during an osmotic challenge presented in aged rats, suggesting that aberration of NOS activity and, by extension, NO signaling, is a potential mechanism of PCB-induced effects.

Materials and Methods

Animals: A total of 22 male Sprague-Dawley rats (ranging in bodyweight from 750-1000g) were utilized for this study. All animals were allowed to age 14 to 16 months in a vivarium with a 12 hour light cycle and *ad libitum* access to food and water. Pregnant dams were fed cheese puff snacks infused with Aroclor 1254 (30mg/kg/d) for 10 days during gestation (GD 10-19). All procedures were performed in accordance and with the approval of the Institutional Animal Care and Use Committee (University of California, Riverside). Dehydration was induced by ip injection of 3.5M NaCl solution (pH 7.4) at doses of 0.6 ml/ 100g body weight followed by water deprivation. Euhydrated rats were given ip injections of isotonic 0.15 M NaCl solution (pH 7.4) at the same doses. A group of dehydrated rats were given 1-2 ip injections of L-NAME, a NOS inhibitor, at doses of 50-100 mg/kg body weight and 30-180 min prior to dehydration. Blood osmolality values for each animal were taken 3-4 hours after saline injection. **Histochemistry:** Animals were anesthetized with low doses of Euthasol (0.08 cc/100g body weight) and sacrificed by transcardial perfusion. The vasculature was cleared of blood using 5mM phosphate-buffered saline (PBS) and tissue was fixed with a 4% paraformaldehyde solution. Brains were removed, blocked and post-fixed in 4% paraformaldehyde for 3 d. Subsequently, brains were cryoprotected in a 30% sucrose solution, and later placed in embedding medium (Tissue-Tek) and frozen at -20°C. Brain blocks were cryosectioned at 40 µm and mounted on gelatin alum-coated slides. Slides were labeled to indicate treatment and rat number then stored at -20°C until staining. **NADPH-diaphorase histochemistry:** Mounted sections were washed 4 X 10 min with ice cold PBS containing 0.1% Triton X-100 (PBS-T). Staining solution was prepared in the absence of light and consisted of 30 mg β-NADPH (SIGMA), 3.0 mg Nitro tetrazolium blue, 0.3% Triton X-100 in 30 ml phosphate buffer. Sections were then bathed in staining solution and incubated at 37°C for 2.0 to 2.5 h. During incubation, staining solution was replenished as needed. Stained sections were allowed to air-dry overnight and dehydrated and cover slipped the following day. **Computer-Assisted Densitometry and Data Analysis:** Stained sections containing the entire SON were imaged with a Nikon TMS light microscope (20X objective) and captured with a Spot Insight color camera. Images were then analyzed using a computer-based densitometry program (Image Pro Plus, Media Cybernetics). SON-region of interest was corrected by subtracting out non-specific background taken from SON sections incubated in the absence of tetrazolium blue. From the corrected images the intensity values were determined by dividing the quotient of sum density versus polygon area by one thousand and presented in arbitrary units. Intensity values for individual animals were pooled into their respective treatment groups. Values for each treatment group are presented as the mean ± SEM. Statistical difference was measured at $p<0.05$ utilizing Student's t-test for groups of two. For multiple group comparisons a one-way analysis of variance (ANOVA) was performed when data met normality and equal variance assumptions. A Dunnett's Test ($\alpha <0.05$; Sigma Stat 2.03) was utilized for *post hoc* comparisons.

Results and Discussion

As shown in Figure 1, microscopic examination revealed that cellular dehydration (Panel B) results in an increase in staining intensity and number of NADPH diaphorase-reactive neurons throughout the nucleus compared to tissue prepared from euhydrated animals (Panel A). *In utero* exposure to Aroclor 1254 (Panel D) reduces the staining

intensity of the osmotically-stimulated SON as does treatment with L-NAME, a pharmacological inhibitor of NOS (Panel C). Employing a computer-assisted image processing system allowed for quantification of the NADPH-diaphorase staining intensity. As shown in Figure 2, SON prepared from hyperosmotically-stimulated PCB-naive rats showed increased NOS activity when compared to euhydrated PCB-naïve rats (6.4 vs. 2.7). In contrast, Aroclor 1254 exposure resulted in a significant decrease in NOS activity in response to the osmotic stimulus (3.8 vs. 6.4). In other words, the osmotic challenge increased NOS activity in control animals by 240% but animals perinatally exposed to the toxicant 15 months prior responded with an increase of only 140%. Dehydrated PCB-naïve animals injected with L-NAME, a pharmacological inhibitor of NOS, showed a downward trend that failed to reach statistical significance ($p>0.05$).

No evidence of necrosis was observed in these sections, suggesting that the PCB effect was not due to acute cytotoxicity. These data suggest that developmental exposure to Aroclor 1254 induces a long-term suppression of

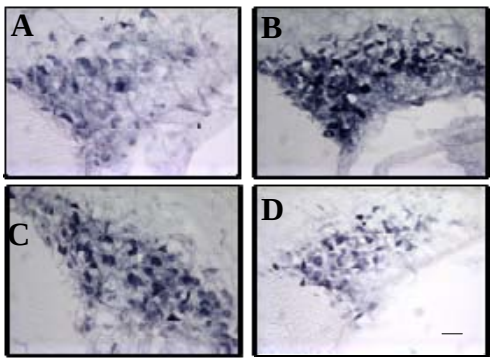

Figure 1. Effect of dehydration and developmental Aroclor 1254 exposure on NADPH-diaphorase staining in the SON of 14-16-month-old rats. Panel A: normosmotic control; Panel B: hyperosmotic control; Panel C: hyperosmotic and L-NAME. Panel D: hyperosmotic and Aroclor 1254. Cal bar = 50 µM.

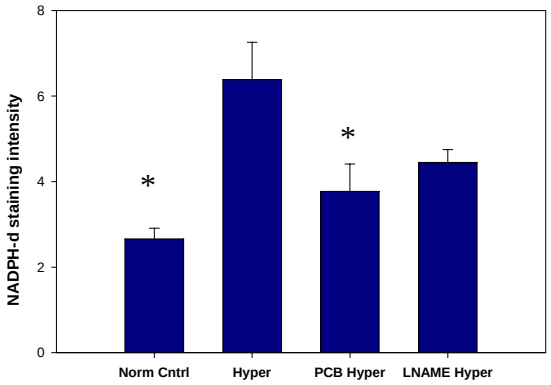

Figure 2. Quantitative changes in NADPH-diaphorase staining intensity in SON of aged rats exposed to Aroclor 1254 *in utero* (30mg/kg/day:GD 10-19). PCB exposure results in a significant decrease in NADPH-d activity during osmotic challenge (Hyper). The number of animals represented by each bar is (from left to right): 4, 6, 7 and 5. *indicates a statistically significant decrease in activity as compared to hyperosmotic group.

osmotically-induced nitric oxide synthase activity in neurosecretory cells of the SON. Current studies are exploring whether these effects are manifested in early adulthood, or if they are only detectable with aging. Others have shown that NAPH-diaphorase staining is selectively elevated within the MNC cell bodies of dehydrated rats and this effect is exaggerated in aged (14-16 month old) animals, suggesting that MNCs in older animals might be more susceptible to NOS inhibition[23,25-26]. Because the modulatory effect of NO on systemic vasopressin release has been demonstrated at both the hypothalamic and posterior pituitary level, and the effects of intranuclear VP and NO appear to exert a largely inhibitory influence on MNC activity and systemic VP release, our current findings might suggest the hypothesis that dampened NOS activity could result in exaggerated systemic hormone output via decreased central VP release in response to an osmotic stimulus[23,27-29]. Over the lifetime of an animal this response could lead to deleterious effects on osmoregulation and cardiovascular functions, such as chronic hypertension and/or hyponatremia, a common symptom associated with congestive heart failure[30]. In the current study we suggest

that perinatal exposure to Aroclor 1254 induces a long-term derangement in NOS activity within the SON. Because central VP release also plays a role in cognitive functions, these findings could apply broadly to the central nervous system and higher brain function.

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References

1. Kodavanti PRS. *Dose-Response* 2005; 3:273.
2. Lang V. *J Chromatogr* 1992; 595:1.
3. She J, Holden A, Sharp M, Tanner M, Williams-Derry C, Hooper K. *Chemosphere* 2007; 67(9):S307.
4. Ellis-Hutchings R, Cherr G, Hanna L, Keen C. *Toxicol Appl Pharmacol* 2006; 215:135.
5. Lilienthal H, Hack A, Roth-Harer A, Grande SW, Talsness CE. *Environ Health Perspect* 2006; 114(2):194
6. Tilson HA, Kodavanti PR, Mundy WR, Busnell PJ. *Toxicol Lett* 1998; 102-103:631.
7. Schuman EM, Madison DV. *Science* 1991; 254(5037):1503.
8. Palumbo ML, Fosser NS, Rios H, Zubilete MA, Guelman LR, Cremaschi GA, Genaro AM. *J Neurochem* 2007;epub ahead of print.
9. Dawson T, Dawson V. *Annu Rev Med* 1996; 47:219.
10. Liberatore G, Jackson-Lewis V, Vukosavic S, Mandir A, Vila M, McAuliffe G, Dawson V, Dawson T, Przedborski S. *Nature Medicine* 1999; 5(12):1403.
11. Sharma R, Kodavanti P. *Toxicol Appl Pharmacol* 2002; 178:127.
12. Canzoniero L, Adornetto A, Secondo A, Magi S, Dell'Aversano C, Scorziello A, Amoroso S, Di Renzo G. *J Neurosci Res* 2006; 84:692.
13. Ludwig M, Leng G. *Adv Exp Med Biol* 1998; 449:163.
14. Ring R. *Curr Pharm Design* 2005; 11:205.
15. Villar MJ, Ceccatelli S, Ronnqvist M, Hokfelt T. *Brain Res* 1994; 644:273.
16. Ueta Y, Levy A, Chowdrey HS, Lightman SL. *Neurosci Res* 1995; 23:317.
17. Gillard ER, Coburn CG, de Leon A, Snissarenko EP, Bauce LG, Pittman QJ, Hou B, Currras-Collazo MC. *Endocrinology* 2007; 148(2):479.
18. Stern J, Zhang W. *J Physiol* 2005; 562(Pt 3):725.
19. Coburn CG, Gillard E, Curras-Collazo MC. *Toxicol Sci* 2005; 84:149.
20. Coburn CG, Curras-Collazo MC, Kodavanti PRS. *Toxicol Sci* 2007; epub ahead of print
21. Matsumoto T, Nakane M, Pollock JS, Kuk JE, Forstermann U. *Neurosci Lett* 1993; 155(1):61.
22. Young HM, O'Brien AJ, Furness JB, Ciampoli D, Hardwick JP, McCabe TJ, Narayanasani R, Masters BS, Tracey WR. *Cell Biol* 1997; 107(1):19.
23. Kadowaki K, Kishimoto J, Leng G, Emson PC. *Endocrinology* 1994; 134:1011.
24. Kishimoto J, Tsuchiya T, Emson PC Nakayama Y. *Brain Res* 1996; 720:159.
25. Pow DV. *J Endocrinology* 1992; 4(4):377.
26. Soinila S, Sadeniemi M, Lumme A, Vanhatalo S. *Neuroscience Lett* 1999; 272:13.
27. Curras-Collazo MC, Gillard ER, Pandika J. *J Neuroendocrinology* 2003; 15(2): 182.
28. Stern J, Ludwig M. Am J Physiol Regul Integr *Comp Physiol* 2001; 280:R1815.
29. Liu H, Terrell ML, Bui V, Summy-Long JY, Kadekaro M. *Physiol Behav* 1998; 63:763.
30. Patel GP, Balk RA. *Clin Ther* 2007; 29(2):211.