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Permanently compromised NADPH-diaphorase activity within the osmotically activated supraoptic nucleus after *in utero* but not adult exposure to Aroclor 1254

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ABSTRACT

Stimulated vasopressin (VP) release from magnocellular neuroendocrine cells in the supraoptic nucleus (SON) of hyperosmotic rats is inhibited by treatment with the industrial polychlorinated biphenyl (PCB) mixture, Aroclor 1254. Because VP responses to hyperosmotic stimulation are regulated by nitric oxide (NO) signaling, we studied NO synthase (NOS) activity in the SON of hyperosmotic rats as potential target of PCB-induced disruption of neuroendocrine processes necessary for osmoregulation. To examine PCB-induced changes in NOS activity under normosmotic and hyperosmotic conditions, male Sprague-Dawley rats were exposed to Aroclor 1254 (30 mg/kg/day) *in utero* and NADPH-diaphorase (NADPH-d) activity was assessed in SON sections at three ages: postnatal day 10, early adult (3–5 months) or late adult (14–16 months). Hyperosmotic treatment increased mean NADPH-d staining density of oil hyperosmotic controls by 19.9% in early adults and 58% in late adulthood *vs* normosmotic controls. *In utero* exposure to PCBs reduced hyperosmotic-induced upregulation of NADPH-d activity to control levels in early adults and by 28% in late adults. Basal NADPH-d was reduced in postnatal rats. Rats receiving PCB exposure as early adults orally for 14 days displayed normal responses. Our findings show that developmental but not adult exposure to PCBs significantly reduces NOS responses to hyperosmolality in neuroendocrine cells. Moreover, reduced NADPH-d activity produced by *in utero* exposure persisted in stimulated late adult rats concomitant with reduced osmoregulatory capacity *vs* oil controls (375 ± 9 *vs* 349 ± 5 mOsm/L). These findings suggest that developmental PCBs permanently compromise NOS signaling in the activated neuroendocrine hypothalamus with potential osmoregulatory consequences.

1. Introduction

Persistent organic chemicals used in industrial applications are major contributors to environmental contamination, and are responsible for contributing to a variety of deleterious human health effects (Carpenter, 2006). Polychlorinated biphenyls (PCBs) are persistent organohalogen compounds of which chronic exposure can lead to endocrine disruption and neurotoxicity (Kodavanti, 2005; Kodavanti and Curras-Collazo, 2010). Although the manufacture of PCBs (including the high production industrial mixture, Aroclor 1254), was discontinued in 1977, PCBs are still found in significant quantities in the environment and human tissues including human breast milk (Lang, 1992; She et al., 2007).

Experimental studies suggest that Aroclor 1254 has the potential to disrupt endocrine functions associated with reproductive and thyroid homeostasis. For example, it has been demonstrated that developmental exposure to Aroclor 1254 significantly decreases circulating total serum thyroxine and testosterone

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levels (Bowers et al., 2004; Crofton et al., 2000; Hany et al., 1999) and postnatal or adult exposure reduces reproductive function in male and female rats (Jonsson et al., 1975; Sager, 1983). In human studies developmental exposure to Aroclor 1254 has been associated with attention and learning deficits in children (Jacobson and Jacobson, 2003) and with abnormal reproductive function and reduced testosterone (Bell, 2014).

Brain function may be affected by neurotoxicants *via* perturbations in intracellular signaling, particularly at critical phases during development (Tilson et al., 1998). Although the specific mechanisms of action responsible for PCB-induced endocrine disruption remain to be fully elucidated, there are several well-documented modes of action associated with PCB-induced neurotoxicity. These modes of action include (1) altered intracellular calcium homeostasis (Inglefield et al., 2001), (2) modulation of PKC (Kodavanti and Ward, 2005), (3) altered glutamate neurotransmission (Mariussen and Fonnum, 2001), and (4) decreased levels of neurotransmitters such as dopamine (Seegal et al., 2002) and serotonin (Khan and Thomas, 2004) in rodent brain. Increasing evidence suggests that nitric oxide (NO) signaling might also be disrupted by PCBs and other endocrine disrupting chemicals (EDCs, Sharma and Kodavanti, 2002; Martini et al., 2010; Currás-Collazo, 2011). NO which is produced by nitric oxide synthase (NOS), is a gaseous neurotransmitter that serves an important role in neuroendocrine function (Garthwaite and Boulton, 1995), in synaptic processes associated with learning and memory such as a retrograde messenger underlying long term potentiation (LTP), (Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991), and in neurodegeneration (Liberatore et al., 1999; Palumbo et al., 2007). Therefore, results from studies on the effect of EDCs on NO signaling can inform significantly about the broad range of adverse effects produced by these neuroactive chemicals.

Magnocellular neuroendocrine cells (MNCs) of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus release vasopressin (VP) both centrally and systemically in response to dehydration, hemorrhage and stress (Ludwig and Leng, 1998; Reid, 1994). *In vivo* studies have shown that intrinsically osmosensitive MNCs receive critical osmosensitive inputs from the central osmosensory nucleus, the organum vasculosum lamina terminalis (OVLT) *via* glutamatergic synapses (Trudel and Bourque, 2012). NO has been described as an activity-dependent modulator of the magnocellular neurosecretory system. We and others have shown that NO signaling is required for stimulated VP release from SON tissue punches (Gillard et al., 2007) and the latter serves to regulate MNC electrical activity and pituitary VP output (Kadowaki et al., 1994; Liu et al., 1998; Stern and Ludwig, 2001; Stern and Zhang, 2005). Moreover, nNOS expression and NOS activity (as assessed by NADPH-d) in MNCs appears to be responsive to physiological state, suggesting an activity-dependent role for NO signaling in the control of body water and salt balance. For example, prolonged dehydration and salt-loading (Ueta et al., 1995) elicits increased nNOS-immunoreactivity as well as increased nNOS expression among SON MNCs, some of which were identified as VP-producing cells.

Data from our lab indicate that PCBs and the structurally related brominated flame retardants, polybrominated diphenyl ethers (PBDEs), significantly reduce physiologically-activated central VP release from rat SON *in vitro* (Coburn et al., 2005, 2007). Given that some ortho-substituted PCB congeners have been reported to inhibit hypothalamic NOS *in vitro* (Sharma and Kodavanti, 2002), the current investigation was designed to determine if PCB exposure *in vivo* alters NOS activity in SON MNCs during osmotic challenge in order to delineate a potential mechanism of neuroendocrine disruption by PCBs. In this study we also examined the differential susceptibility of NOS activity within the SON to developmental and adult exposure to PCBs.

To this end we used nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase (NADPH-d) histochemistry. NADPH is a required cofactor for NOS enzyme activity (Dawson et al., 1991) and NOS has NADPH-d activity (Hope et al., 1991). An enhancement of NADPH-d staining in fixed sections coincides with a respective rise in the amount of NOS protein (Kishimoto et al., 1996) and NOS gene expression (Kadowaki et al., 1994); therefore, NAPDH-d staining can be used as an indicator of NOS activity in paraformaldehyde-fixed tissue (Matsumoto et al., 1993; Young et al., 1997). Our data indicate that early developmental PCB exposure significantly compromises NOS responses to osmotic activation in the adult supraoptic nucleus, an effect which may lead to PCB-induced disruption of neuroendocrine function and osmoregulation.

2. Materials and methods

2.1. Animals; exposure to Aroclor 1254 and osmotic activation

Male Sprague-Dawley rats were purchased from Charles River Laboratories and maintained in a vivarium with a 12-hour light cycle and *ad libitum* access to food and water. All procedures were performed in accordance and with the approval of the Institutional Animal Care and Use Committee (University of California, Riverside). Four cohorts of rats were studied: (1) adult male rats dosed as adults (480–691 g, 3–5 months old), (2) developing pups dosed *in utero* (19–26 g, PND 10), (3) adult male rats dosed *in utero* (492–799 g, 3–5 months old), (4) late adulthood male rats dosed *in utero* (412–933 g, 14–16 months old).

A single lot (# 124-191-B) of Aroclor 1254 (purity >99%; AccuStandard, Inc., New Haven, CT) was used for these studies. Oral exposure to Aroclor 1254 was accomplished by giving cheese puff snacks (Cheeto©) infused with Aroclor 1254 daily (30 mg/kg/day) in addition to their normal diet. Toxicant delivery *via* cheese puff snacks was first established in our lab and has proven to serve as a successful method of exposure for PCBs and PBDEs (Coburn et al., 2005; Shah et al., 2011). Exposure of adults (3–5 months old) occurred for 14 days; the final dose was given the night prior to the day of sacrifice. *In utero* exposure was accomplished by feeding pregnant dams cheese puff snacks infused with Aroclor 1254 (30 mg/kg/day) for 10 days during gestation (GD 10–19). Post parturition, pups were allowed to suckle on the dam until weaning at postnatal day 22 (PND 22). Some pups were culled and examined at PND 10 ($n = 14$). The remaining male pups were allowed to mature and were either sacrificed at 3–5 months of age ($n = 72$) or allowed to age for 14–16 months before sacrifice ($n = 48$). Corn-oil dosed rats served as vehicle controls. Upon completion of the dosing regimens, animals were randomly assigned to receive a hyperosmotic challenge or remain in the normosmotic state. In adult rats intracellular brain dehydration was induced by intraperitoneal (i.p.) injection of hyperosmotic saline (3.5 M NaCl; pH 7.4) at doses of 0.6 ml/100 g b.w. followed by water withholding. Euhydrated (normosmotic) rats received an injection of isotonic solution (0.15 M NaCl; pH 7.4, i.p.). Drinking water was removed from hyperosmotic rats for 3–4 h until sacrifice. To compare reductions in NADPH-d activity produced by PCB exposure to that produced by acute NOS blockade, a group of control hyperosmotic rats were injected with N^G-nitro-L-arginine-methyl ester (L-NAME), a general NOS inhibitor (Rees et al., 1990), at a dose of 50 mg/kg, i.p. at 30 min prior to and 1.5 h following the *in vivo* saline injection. Plasma osmolality for each animal was measured in blood taken at sacrifice. Pooled values for all groups are shown in Table 1. Control (PCB-naïve) animals in the physiological saline group for all adult cohorts displayed normal baseline plasma osmolalities (288–300 mOsm/kg) and are referred to subsequently as *normosmotic* control animals.

Table 1
Osmoregulatory capacity measured during acute hyperosmotic stress in rats exposed to Aroclor 1254 as adults or *in utero*. Plasma osmolality values (mean ± S.E.M.) were measured 3–4 h following i.p. injection (0.6 ml/100 g b.w.) of normosmotic saline (0.15 M NaCl; Normal) or hyperosmotic saline (3.5 M NaCl; Hyper) in three cohorts of rats. In the first cohort early adult (3–5 months) male Sprague-Dawley rats were given oral cheetoh snacks infused with the industrial PCB mixture Aroclor 1254 (30 mg/kg/day × 14 day) in corn oil vehicle (PCB) and osmotically treated on day 15. The second and third cohorts were exposed to Aroclor 1254 *in utero* (30 mg/kg/day; GD 10–19) and osmotically treated in early adulthood (3–5 months) or in late adulthood (14–16 months), respectively. PCB-naive rats were exposed to corn oil vehicle and did not receive PCBs (Oil). Some hyperomsotic Oil rats received acute treatment with the NOS blocker L-NAME (50 mg/kg X 2, i.p.; Hyper + L-NAME). * Indicates statistical difference *vs* Hyper Oil, $p < 0.05$. # Indicates statistical difference between Hyper + PCB *vs* Hyper Oil; $p < 0.05$.

Age of PCB exposure; Observation period	Normal Oil (*n*)	Normal + PCB (*n*)	Hyper oil (*n*)	Hyper + PCB (*n*)	Hyper + L-NAME (*n*)
PCB early adult; Early adult	294.3 ± 6* (3)	292.3 ± .8* (4)	345.0 ± 8 (4)	339.8 ± 9 (6)	–
PCB *in utero;* Early adult	297.5 ± 2* (4)	–	342.1 ± 8 (4)	355.2 ± 4 (7)	360.0 ± 19 (3)
PCB *in utero;* Late adult	297.7 ± 4* (6)	–	348.6 ± 5 (6)	375.0 ± 9# (7)	365.3 ± 14 (5)

2.2. NADPH-diaphorase histochemistry

Rats were sacrificed under deep anesthesia with sodium pentobarbital (100 mg/kg) and the brains fixed by transcardial perfusion. First, the vasculature was cleared of blood using ice-cold, heparinized, 0.01 M phosphate buffered saline (PBS) and the brain was fixed *in situ* with a 4% phosphate-buffered paraformaldehyde solution. Brains were removed, blocked and post-fixed in 4% paraformaldehyde for 3 h for PND 10 animals and 3 days for all others. Subsequently, brains were cryoprotected in 30% sucrose solution, later placed in embedding medium (Tissue-Tek©), cryosectioned at 40 μm and sections containing the SON were mounted on gelatin alum-coated slides which were then stored at −20 °C until processed using NADPH-d histochemistry. Mounted sections were washed with ice-cold PBS containing 0.1% Triton X-100 (PBS-T). Sections were then incubated in staining solution containing 30 mg β-NADPH (Sigma), 3.0 mg nitro tetrazolium blue (Sigma) in PBS-T at 37 °C for 2–2.5 h in the dark. After staining, sections were washed with cold TRIS buffer (50 mM; pH 7.4), air-dried overnight, dehydrated in alcohol, delipidated and cover-slipped. A subset of samples were subjected to *in vitro* exposure to the competitive NOS inhibitor, N^G-nitro-L-arginine (L-NNA; Sigma; 10 mM). This was undertaken for 1 h prior to, or during the entire staining reaction. Both protocols yielded similar reductions of NADPH-d staining density.

2.3. Computer-assisted densitometry

Stained sections containing the entire SON were analyzed under a light microscope (TMS, Nikon) and images captured with a digital color camera (Diagnostic Instruments). Images were then analyzed using a computer-based densitometry program (Image Pro Plus, Media Cybernetics). Images were acquired of the body of the SON region excluding the ventral glial limitans (3 per rat) and corrected by subtracting out non-specific background color determined using SON sections incubated in the absence of β-NADPH. From the corrected images the intensity values were determined by dividing the sum density of staining by the polygon area (area of the SON) and dividing by one thousand. Density values were expressed as % control values obtained from hyperosmotic rats (receiving no drugs or toxicants). Density values were then pooled for each treatment group.

2.4. Statistical analysis

Unless otherwise noted the mean NADPH-d staining density for the hyperosmotic group served as the control value (100%), and the mean value for each treatment was expressed as a percentage of control value to eliminate any variability in methodology between experiments. For two-group comparisons, statistical difference was measured using a Student's *t*-test. For multi-group comparisons, a general linear model ANOVA was used where data met normal distribution/equal variance assumptions; otherwise Kruskal–Wallis ANOVA on Ranks was used. Where overall significance ($p < 0.05$) was obtained, *post hoc* multiple comparisons were used to detect specific differences, with Student–Neuman–Keuls test applied following general linear model ANOVA and Dunn's test applied following ANOVA on Ranks. An α level of 0.05 was used to indicate statistical significance.

3. Results

3.1. Hyperosmotic stimulation enhanced neuronal NOS activity in SON

MNCs in the SON showed intense NADPH-d staining that was enhanced after a hyperosmotic challenge in both early and late adults. As a result of cellular dehydration in the brain *in vivo*, striking differences in the NADPH-d staining density within the SON can be observed between normosmotic (Norm) and hyperosmotic (Hyper) animals (Fig. 1). To quantify these observations, mean NADPH-d staining density for the hyperosmotic group served as the control value (100%), and the mean value for each

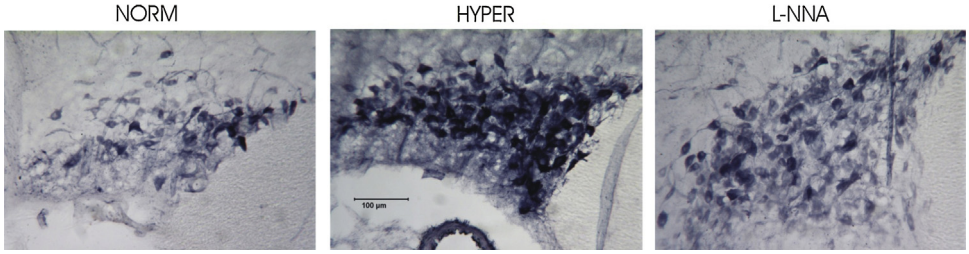

Fig. 1. Enhanced neuronal NADPH-d activity in the SON in response to acute hyperosmotic stimulation is representative of upregulated NOS activity. Early adult male Sprague-Dawley rats were subjected to either normosmotic (0.9 g% NaCl; Norm) or hyperosmotic (3.5 M NaCl, Hyper) injection (0.6 ml/100 g b.w; i.p.). Rats were sacrificed 3–4 h later by transcardial perfusion under deep anesthesia. Brains were processed for NADPH-d staining in the presence or absence of the NOS blocker, N^G-nitro-L-arginine (L-NNA; 10 μM). Images show representative stained SON sections obtained from normosmotic (NORM) and hyperosmotic rats (HYPER). Calibration Bar = 100 μm.

treatment was expressed as a percentage of control value. Fig. 1 shows that hyperosmotic stimulation results in a significant increase in NADPH-d staining in dehydrated rat SON when compared to age-matched normosmotic control animals (100.0 ± 11.5 *vs* $42.0 \pm 4.2\%$; $n = 11$; $p < 0.01$), representing a 58% increase in NADPH-d activity over normosmotic values. An established irreversible competitive inhibitor of NOS *in vitro*, L-NNA (Blottner and Baumgarten, 1995; Michel et al., 1993), significantly reduced the enhanced staining seen in the hyperosmotically activated SON when added directly to the fixed tissue. When compared to hyperosmotic control SON, hyperosmotic L-NNA-treated (10 mM) tissue showed significantly lower mean staining density, respectively ($100.0 \pm 11.6\%$ *vs* 45.3 ± 9.7; $n = 13$; $p < 0.05$). Therefore, L-NNA suppressed hyperosmotically stimulated enhancement of NADPH-d staining density to levels that are statistically indistinguishable from normosmotic control animals ($42.0 \pm 4.2\%$), confirming that NADPH-d staining is representative of NOS activity (Blottner and Baumgarten, 1995). Constitutive NADPH-d staining in the hyperosmotic plus L-NNA condition (as well as in normosmotic condition) may represent insensitive isoforms of NOS (iNOS; Reif and McCreedy, 1995) other than nNOS, for which L-NNA has the highest affinity ($K_i = 25$ nM).

3.2. Adult exposure to Aroclor 1254 has no effect on activity-dependent NOS activity

Upon completion of the PCB dosing during adulthood, early adult rats were randomly assigned to receive a hyperosmotic challenge or remain in the normosmotic state. After 3–4 h rats were sacrificed and their brains processed for NADPH-d histochemistry. Fig. 2 shows a significant increase in NADPH-d staining density in SON sections obtained from hyperosmotic PCB-naïve rats (Hyper Control) as compared to normosmotic PCB-naïve animals (Normal Control). Normalized staining density values obtained for hyperosmotic and normosmotic control rats were 100.6 ± 5.1 and $80.5 \pm 6.0\%$, respectively ($n = 39$; $p < 0.01$). Importantly, Fig. 2 shows no significant effect of dietary PCB exposure between hyperosmotic PCB-treated rats (Hyper PCB) and hyperosmotic PCB-naïve controls, respectively (107.6 ± 12.3 *vs* $100.6 \pm 5.1\%$; $n = 29$).

3.3. Prenatal Aroclor 1254 reduces basal NOS activity in the SON

Pups exposed *in utero* to PCBs or corn oil vehicle were sacrificed at PD10 and processed for NADPH-d histochemistry. Because these pups were still suckling, no osmotic challenge or i.p. injection was administered to this experimental cohort. As shown in Fig. 3(Top), *basal* NADPH-d staining density was markedly lower in SON sections from pups exposed to Aroclor 1254 (C) as compared to PCB-naïve oil controls (A). Panels B and D show magnified micrographs of the SON areas shown in A and C, respectively. Pooled optical density values for Aroclor 1254 (A1254) and oil vehicle groups (control) are shown in Figure 3 (Bottom). Mean NADPH-d staining density values were 76.8 ± 5.4 for PCB-exposed pups (A1254) *vs* $100 \pm 5.8\%$ for PCB-naïve pups. Therefore, ontogenic exposure to Aroclor 1254 resulted in a statistically significant reduction of 23% in NADPH-d staining density in SON compared to PCB-naïve control sections during basal conditions ($p < 0.01$).

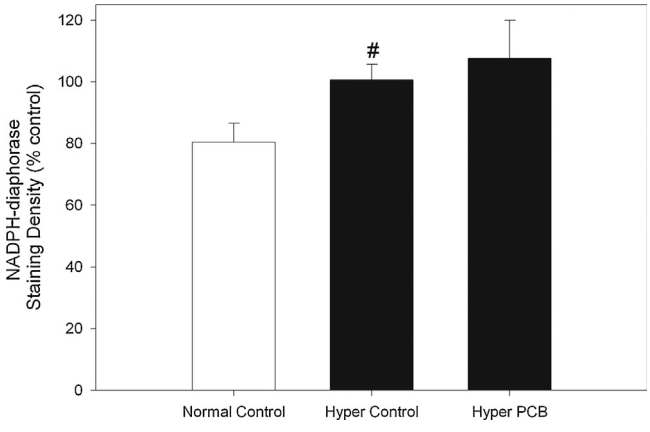

Fig. 2. Lack of effect of adult exposure to Aroclor 1254 on activity-dependent NOS activity within the SON. Bars represent pooled values of mean NADPH-d staining intensity in the SON of PCB-naïve (control), or PCB-treated male rats fed Aroclor 1254 (30 mg/kg/day × 14 day) and injected with either physiological saline (Normal) or hyperosmotic saline (Hyper). Hyperosmotic stimulation produced an increase in staining density as compared to normosmotic conditions (Hyper Control *vs* Normal Control). # Indicates a significant increase over normal control values ($p < 0.05$). The number of rats comprising each group is (from left to right): 15, 24 and 5.

3.4. Prenatal Aroclor 1254 compromises physiological activation of NOS

In additional experiments rats were exposed *in utero* to Aroclor 1254 and treated with osmotic challenge during early adulthood. As shown in Fig. 4, hyperosmotic stimulation resulted in a significant increase in NADPH-d staining intensity in SON MNCs. Mean values for NADPH-d density were 100.4 ± 3.7 *vs* $80.4 \pm 6.0\%$ (Mann–Whitney Rank sum test, $T = 232.0$, $n = 50$; $p < 0.01$) for hyperosmotic control (Hyper Control) and normal control animals (Normal Control), respectively. In contrast, hyperosmotic-induced NOS upregulation was significantly attenuated in animals prenatally exposed to Aroclor 1254 (Kruskal–Wallis ANOVA on ranks, $H = 19.47$, $df = 2$ followed by Dunn's test, $Q = 4.35$, $p < 0.05$). NADPH-d staining density was reduced by approximately 36% in hyperosmotic PCB-treated animals (Hyper PCB) as compared to PCB-naïve hyperosmotic controls (63.7 ± 5.5 *vs* $100.4 \pm 3.7\%$; $n = 45$). Therefore, prenatal exposure to PCBs effectively lowered the staining density to normosmotic control levels (Fig. 4). These results suggest that prenatal exposure to Aroclor 1254 attenuates the NOS response of the activated SON in early adulthood, an effect that is consistent with the reduced basal NOS activity seen during the postnatal period.

3.5. Persistence of reduced NOS responsiveness in late adulthood rats prenatally exposed to Aroclor 1254

In these experiments rats were exposed to Aroclor 1254 prenatally, allowed to reach late adulthood (14–16 months of age) and then subjected to acute hyperosmotic stimulation and sacrificed 3–4 h later. Microscopic evaluation revealed that cellular dehydration in the brain results in an increased NADPH-d staining density in the SON of aged animals, findings that are consistent with those of others (Soinila et al., 1999; Yun et al., 2005). As shown in Fig. 5, the staining density of NOS-activated neurons throughout the SON is dramatically increased in late adulthood rats subjected to acute hyperosmotic stimulation (Hyper Control; Fig. 5B) compared to normosmotic controls (Normal Control; Fig. 5A). When results from 10 experiments were pooled and analyzed quantitatively (Fig. 6), mean values obtained for NADPH-d density were calculated as 42.4 ± 3.1 *vs* $100 \pm 0.5\%$ for normosmotic and hyperosmotic control animals, respectively ($n = 20$; $p < 0.01$), indicating a 57.6% rise in NOS activity in response to hyperosmotic stress. Mean values for NADPH-d density were 72.0 ± 8.9 *vs* $100 \pm 0.5\%$ for hyperosmotic PCB-treated animals (Hyper PCB) and hyperosmotic controls, respectively, indicating 28% weaker NADPH-d activity in exposed rats (Kruskal–Wallis ANOVA on ranks ($H = 16.58$, $df = 2$) followed by Dunn's test, $Q = 11.8$, $n = 20$, $p < 0.05$).

To examine if acute NOS blockade could reduce net NOS activity to the same extent as Aroclor 1254 a separate group of osmotically stimulated animals were injected with L-NAME. Stimulated NOS activity, measured in rats acutely exposed to L-NAME, decreased markedly by 35.7% (Kruskal–Wallis ANOVA on ranks

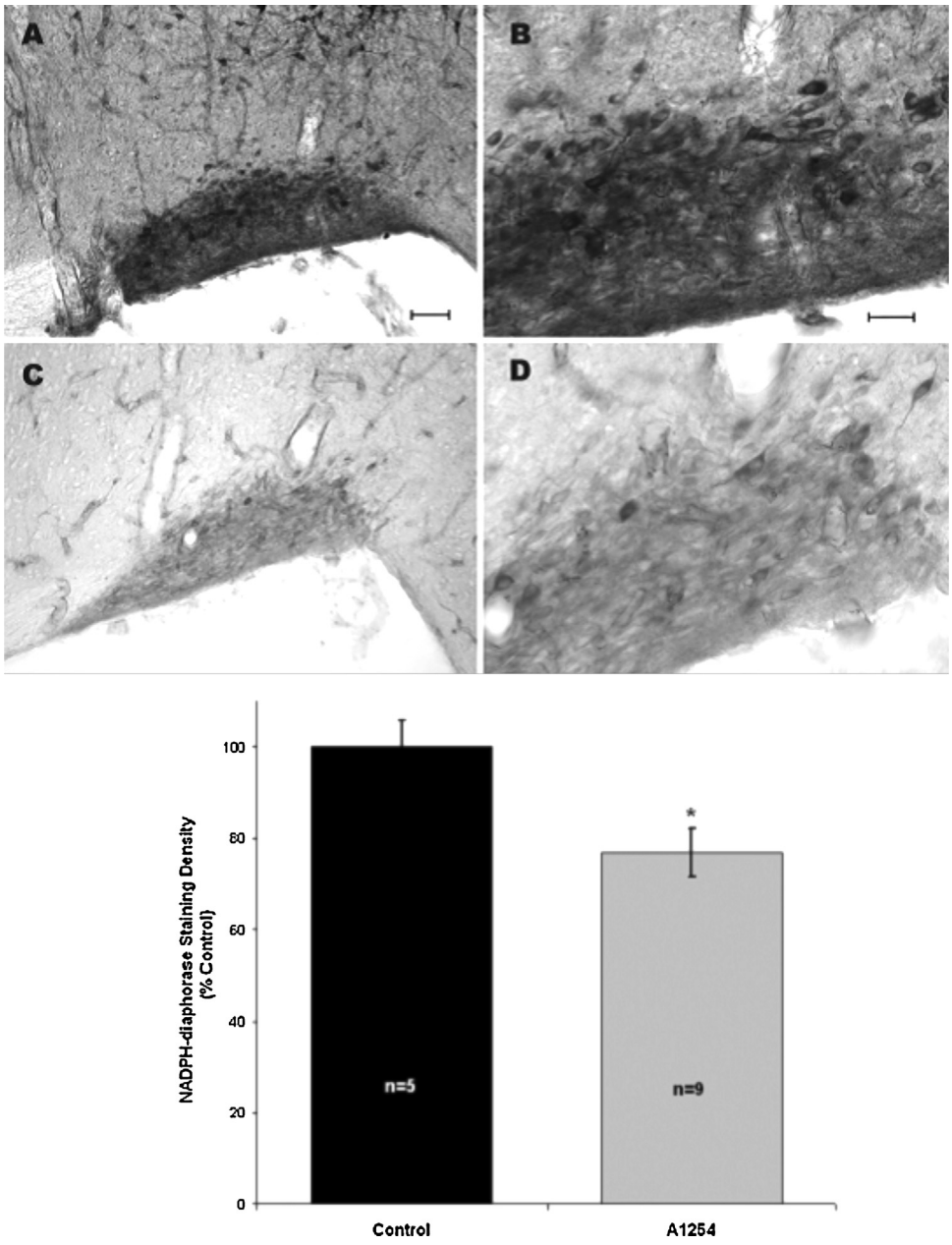

Fig. 3. In utero exposure to Aroclor 1254 disrupts basal NOS activity in the SON during postnatal development. Rat pups were exposed to Aroclor 1254 in utero (30 mg/kg/day; GD 10–19) and were sacrificed at PD10. TOP: Representative photomicrographs showing NADPH-d activity in paraformaldehyde-fixed coronal sections (40 μm) from the SON of juvenile rats born from pregnant dams fed corn oil (A; Control) or dams exposed to Aroclor 1254 (C; A1254). B and D: magnification of the SON area of A and C, respectively. Calibration Bar = 50 μm for A and C; 25 μm for B and D. BOTTOM: Bars represent average NADPH-d staining densities in SON sections from control and A1254 rats. * Indicates a significant decrease in staining intensity in the SON of Aroclor-exposed compared to control rat pups ($n = 14$; $p < 0.01$).

($H = 16.58$, $df = 2$) followed by Dunn's test, $Q = 15.48$, $n = 21$, $p < 0.05$). Mean staining density values were 64.3 ± 3.8 *vs* $100 \pm 0.5\%$ for L-NAME and hyperosmotic controls, respectively (Fig. 6).

We then examined the effect of Aroclor 1254 exposure on NOS activity in the normosmotic condition. As shown in Fig. 7, *in utero* exposure to Aroclor 1254 (normal PCB) resulted in a significant increase in NOS activity in 14–16 month old rats examined under normosmotic conditions (Normal Control; $t = 4.26$, $df = 12$, $p < 0.001$). Surprisingly, basal NADPH-d activity was more than double in these PCB-treated animals (Normal PCB) compared to that of age-matched PCB-naïve normosmotic controls (255.3 ± 35.7 *vs* $100 \pm 7.3\%$; $n = 14$).

3.6. Altered osmoregulation in rats developmentally exposed to Aroclor 1254

PCB-exposed rats and oil controls were injected (i.p.; 0.6 ml/100 g b.w.) with either 3.5 M NaCl (to induce hyperosmotic challenge) or 0.15 M NaCl (physiological saline control) and water was withheld until the animals were sacrificed 3–4 h later. To confirm the effectiveness of the hyperosmotic stimulus, tail blood was collected from each animal and the osmolality of the plasma fraction was measured (Table 1).

Administration of the hyperosmotic stimulus resulted in a statistically significant rise in mean plasma osmolality for oil hyperosmotic control (342–349 mOsm/kg; $p < 0.05$) animals as

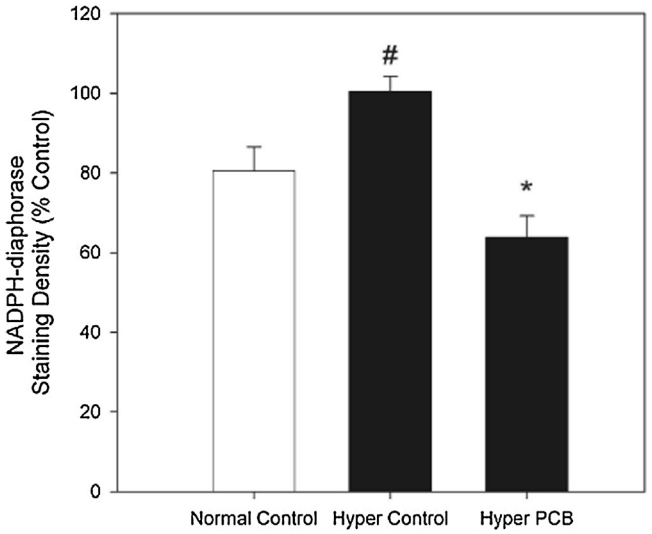

Fig. 4. Prenatal exposure to Aroclor 1254 compromises physiological activation of NOS activity in the SON of early adult rats. NADPH-d staining density was measured in the SON of male adult (3–5 months) PCB-naïve and PCB-treated rats (Aroclor 1254; 30 mg/kg/day; GD 10–19) injected with either physiological saline (Normal Control) or hyperosmotic saline (3.5 M NaCl; 0.6 ml/100 g b.w., Hyper Control). # Indicates a statistically significant increase over normal control values ($p < 0.05$). * Indicates a statistically significant decrease in hyperosmotic Aroclor 1254-exposed rats compared to hyperosmotic control animals (Hyper PCB, $p < 0.05$). The number of rats comprising each group is (from left to right): 15, 35, 10 and 12.

described previously (Gillard et al., 2007). Table 1 shows a general lack of effect of PCB exposure on plasma osmolality values obtained from normosmotic and hyperosmotic rats with the exception of those prenatally exposed to Aroclor 1254 and tested in late adulthood (14–16 months). As a result of the hyperosmotic stimulus, older adult animals showed osmolalities that were 7.5% higher as compared to their respective hyperosmotic controls (375 ± 89 *vs* 348.6 ± 5.2 mOsm/kg; $n = 13$; $p < 0.05$). By comparison, mean plasma osmolality in the hyperosmotic oil animals (early or late adults) receiving acute administration of the general NOS inhibitor, L-NAME, was not significantly changed relative to hyperosmotic controls.

4. Discussion

The NADPH-diaphorase (NADPH-d) enzyme activity that is associated with NOS activity sustains routine fixation procedures with aldehydes and has been used as a reliable indicator of NOS enzymatic activity in paraformaldehyde-fixed brain tissue including the SON (Kadowaki et al., 1994; Matsumoto et al., 1993; Villar et al., 1994). In agreement with others, we found that hyperosmotic stimulation of early and late adulthood rats triggered an activity-dependent increase in NADPH-d staining density in the SON (Soinila et al., 1999; Yun et al., 2005). The increased NADPH-d staining is likely representative of neuronal NOS (nNOS) since NADPH-d has been shown to colocalize with nNOS (Dawson et al., 1991; Hope et al., 1991) but less reliably with endothelial NOS (eNOS) (Dinerman et al., 1994) and inducible NOS (iNOS) (Tracey et al., 1993). In support of this we show that stimulated NADPH-d staining is suppressed by *in vitro* treatment with the general NOS inhibitor, L-NNA, with highest affinity for nNOS ($K_i = 25$ nM) and whose binding sites in the rat CNS correspond well with anti-NOS immunohistochemical staining (Kidd et al., 1995; Reif and McCreedy, 1995).

Here, we report that PND 10 rats exposed to Aroclor 1254 *in utero* show a modest but significant reduction in SON NOS activity, suggesting that developmental exposure to PCBs may compromise NO-dependent MNC functions beginning early in life. Since NO participates in synaptic plasticity, neurite outgrowth and extension, synaptogenesis, neuronal proliferation and maturation (Holscher, 1997; Sanchez-Islas and Leon-Olea, 2004; Wu et al., 1994), the early PCB-induced changes in NOS activity may be associated with altered nervous system development and function (Kodavanti, 2005). Others have suggested that PCB-induced disruption of thyroid hormone signaling in PCB-exposed animals may explain the long-term effects of developmental PCB exposure on nervous system structure and function (Yang et al., 2009). Indeed, perinatal exposure to PCB significantly lowers circulating thyroid hormone levels in rodents (Crofton et al., 2000; Zoeller and Crofton, 2000) and maternal exposure to PCBs is associated with an elevation in thyroid and thyroglobulin antibodies that can pass through the placenta and interfere with thyroid status in the fetus (Langer et al., 1998). Moreover, NOS gene expression in SON MNCs appears to be regulated by thyroid status (Ueta et al., 1995). Therefore, *in utero* exposure to PCBs may be directly toxic to developing hypothalamic nitrergic systems and/or may work indirectly *via* immunomodulatory processes involving the mother (Koppe, 2004) and these effects may involve dysregulation of hypothalamo-pituitary-thyroid axis.

We also report that upregulation of NOS activity in the SON after hyperosmotic stimulation is markedly attenuated in the SON of rats exposed to Aroclor 1254 during development but not in rats orally exposed in adulthood. The more profound effects of *in utero vs* oral adult Aroclor 1254 treatment paradigms cannot be ascribed to differential penetration of PCB congeners depending on age at time of exposure. In fact, a review of reports using exposure paradigms, similar to those employed here, indicates that *in utero* exposure gives rise to lower PCB concentrations in rat pup brains as compared to adult exposure. Individual congeners comprising Aroclor 1254 readily accumulate in brain tissue as a result of an oral dosing regimen given to adult rats (30 mg/kg/day; 5 day/wk × 4wk). This dosing schedule resulted in total PCB levels of 15 and 30 ppm in frontal cortex and liver, respectively, including PCB congeners 138 and 153 (Kodavanti et al., 1998). These are the predominant congeners found in human breast milk sampled from first-time mothers living in the U.S. and Canada (She et al., 2007). In contrast, prenatal dosing as performed by Crofton's group (dosed primiparous dams with Aroclor 1254 at 6 mg/kg/day; GD 6-PND 21) yielded a total PCB value of 3.0 ppm in frontal cortex of postnatal pups (Crofton et al., 2000); these values are 5- to 10-fold lower than those seen after adult exposure.

The enhanced effects of PCBs, when given to developing rats, observed on osmotic regulatory indicators in our study, indicates the critical nature of exposure timing. Indeed, experimental animals appear to be especially sensitive to PCB exposure during the postnatal period as manifested by levels of thyroxine, which are similarly reduced after *in utero* followed by postnatal dosing (perinatal; Crofton et al., 2000; Goldey et al., 1995; Shah et al., 2011). In support of this, others have shown that perinatal dosing can alter levels of male reproductive steroids in adulthood (Hany et al., 1999). In combination with those of others, our findings indicate that gestational exposure to PCBs can promote enhanced endocrine disruption even in spite of potentially lower body burdens than *via* adult exposure. In our study, we show that perinatal treatment with PCBs yields more significant aberrations of plasma osmolality than adult treatment with PCBs. The *in vivo* doses used here are higher than the expected daily toxicant intake in nature by necessity in order to permit a short-term dose regimen to drive PCB concentrations in rat brain to levels reached over the course of a lifetime of bioaccumulation. For comparison, a total average PCB concentration of 148 ppb (0.3 μM) has been detected in the brains of top predators such as East Greenland polar bears

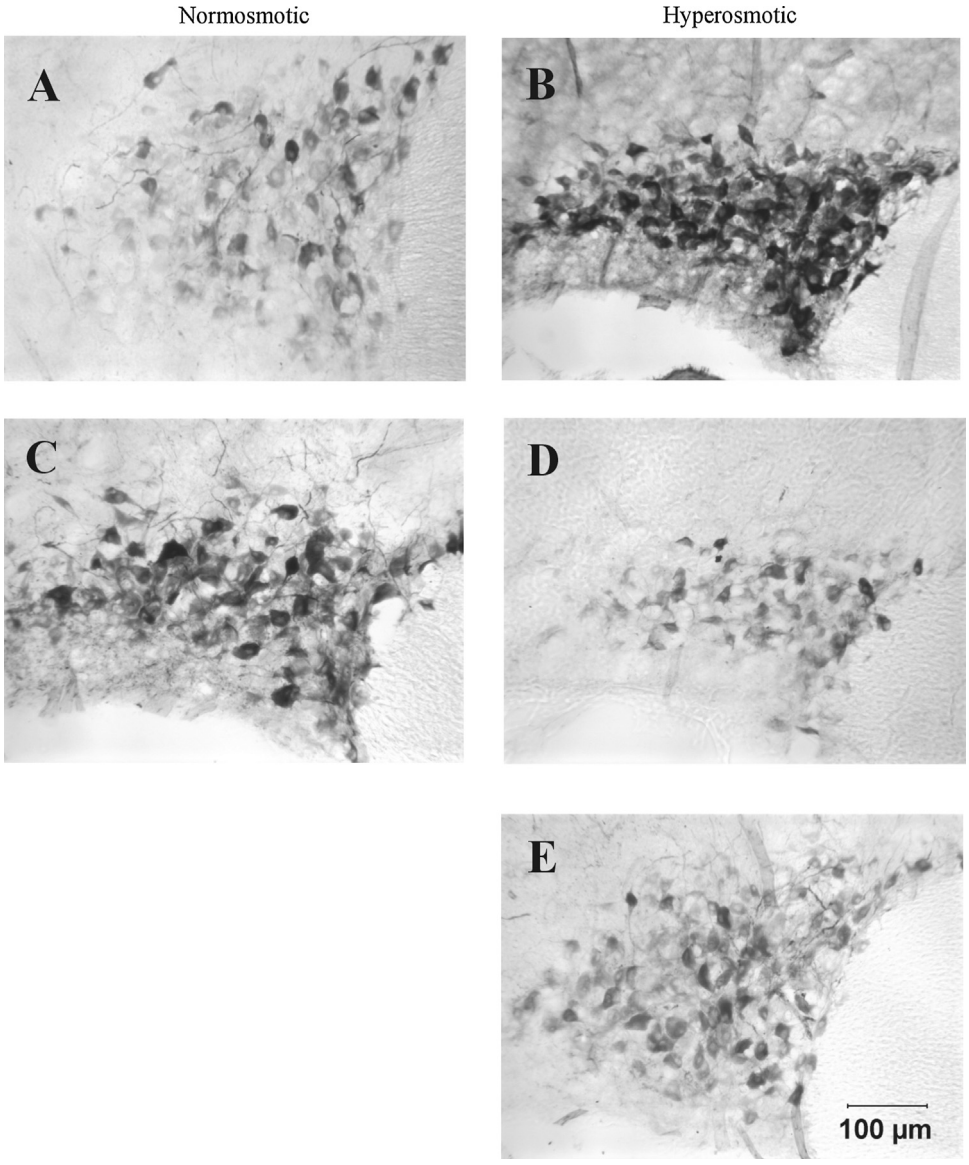

Fig. 5. Persistent long-term effects of prenatal exposure to Aroclor 1254 on activity-dependent NOS activity within the SON. Representative micrographs show NADPH-d staining density in the SON of late adult (14–16 month) male rats injected with either physiological saline (Normosmotic, A,C), or hyperosmotic saline (B,D; 3.5 M NaCl; 0.6 ml/100 g b.w.), or hyperosmotic saline with L-NAME (50 mg/kg, E). Aroclor-treated rats are shown in Panels C and D. Calibration Bar = 100 μm.

(Gebbink et al., 2008a,b). Similarly, elevated levels of summated PCBs (96 ppb; 0.2 μM) have been reported in human breast milk (She et al., 2007) and adult brain levels of 58 ppb (0.1 μM) have been measured in the high-risk population of Inuit Greenlanders (Dewailly et al., 1999).

Reduced NOS activity levels seen in both early and late adulthood rats exposed to PCBs *in utero* may underlie altered neuroendocrine output of VP from dendrites and axons of MNCs, a process that we and others have demonstrated is modulated by NO (Gillard et al., 2007). Such neuroendocrine and osmotic alterations provoked by developmental PCB exposure are long-lived. Developmental PCB exposure also significantly reduced hyperosmotic-induced NADPH-d activity in late adulthood rats in a manner similar to that found for early adults dosed during the same developmental period, but osmotically challenged earlier in life (Fig. 4). These results suggest that the neurotoxic effects of PCB exposure to the developing nervous system may be persistent and potentially maladaptive, and are in agreement with reports demonstrating that PCB exposure during gestation produces long-term neurotoxicity in various behavioral and neurological parameters in rats and mice (Meerts et al., 2004; Ulbrich and Stahlman, 2004). Moreover, the aged hypothalamo-neurohypophysial system (HNS) may be additionally susceptible to developmentally-reduced NOS activity since the NOS response to physiological stimulation is more vigorous in aged rats as shown previously in salt-loaded animals (Soinila et al., 1999). This is consistent with hypertrophied VP-producing MNC somata, Golgi apparatus and rising plasma VP concentrations in aged rodents (Hoogendijk et al., 1985; Lucassen et al., 1994; Sladek and Olschowka, 1994). These results indicate that aging processes may aggravate the negative effects of developmental PCB exposure.

Our previous work has shown that early adult exposure to Aroclor 1254 disrupts osmotically stimulated plasma and central VP (Coburn et al., 2005), an effect that cannot be ascribed to disrupted NADPH-d enzymatic activity as determined in our current study. While we did not measure VP status in this study, our findings demonstrate that osmoregulatory processes are compromised in late adult hyperosmotic rats exposed to Aroclor 1254 *in utero*. These rats show significantly elevated plasma

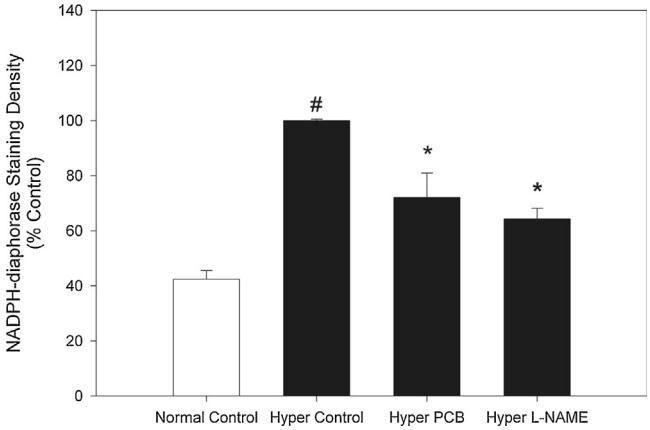

Fig. 6. Prenatal exposure to Aroclor 1254 suppresses hyperosmotic-induced NOS activity in the SON of late adulthood rats. Bars represent NADPH-d staining intensity obtained using SON sections of late adulthood male rats receiving PCBs (Aroclor 1254; 30 mg/kg/day; GD 10–19) or corn oil vehicle (control). Rats were injected with either physiological saline (Normal Control) or 3.5 M NaCl (Hyper Control). # Indicates a significant increase over normal control ($p < 0.05$). * Indicates a significant decrease in staining intensity as compared to hyperosmotic control, $p < 0.05$. The number of rats comprising each group is (from left to right): 10, 10, 10 and 11.

osmolality measured 3–4 h after acute hyperosmotic challenge (Table 1). This was only observed in late adulthood rats, suggesting that physiological regulatory systems controlling body water homeostasis may be compromised only in the aged, hyperactive SON. Therefore, while neuroendocrine outcomes of PCB exposure occur after both *in utero* and early adult exposure to Aroclor 1254, the latter does not lead to abnormal stimulated plasma osmolality responses, an effect that may be due to other targets of PCBs such as intracellular events including Ca^{2+} buffering (Kodavanti and Ward, 2005).

In human studies high body burdens of PCBs and other organohalogen toxicants have been correlated with elevated basal levels of plasma VP and blood pressure in humans (Jorgensen et al., 2003; Kreiss, 1985; Kreiss et al., 1981). Interestingly, late adulthood rats displayed exaggerated NADPH-d activity in the normosmotic PCB group relative to normosmotic controls (Fig. 7), a result which may explain hyperactive neuroendocrine function in the absence of osmotic activation. These observations, which differ

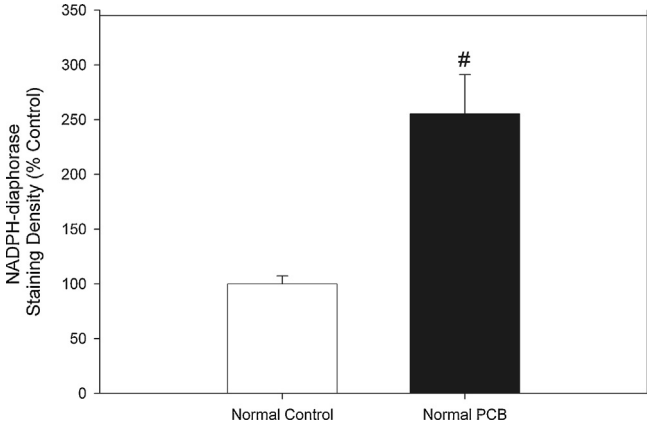

Fig. 7. Prenatal exposure to Aroclor 1254 upregulates basal NOS activity in the SON of late adulthood rats. Bars represent pooled NADPH-d staining densities for SON of normosmotic male PCB-naïve (Normal Control) and normosmotic male rats previously exposed to Aroclor 1254 prenatally (30 mg/kg/day, GD 10–19; Normal PCB). Rats were injected i.p. in late adulthood (14–16 months) with normosmotic saline (0.9 g% NaCl, 0.6 ml/100 g b.w.). # Indicates a statistically significant difference when compared to Normal Control ($p < 0.001$; $n = 7$ per group).

from the inhibitory effect observed during hyperosmolality, support the concept that PCBs may exert differential effects in the same animal depending on the physiological (and developmental) state. In combination, these findings indicate a potential maladaptive effect of developmental PCB exposure on the HNS that may translate into compromised physiology related to the hormone VP, such as osmoregulation and cardiovascular function, in part, *via* actions on NO.

In agreement with the findings by others (Ayers et al., 1997), using a similar dosing regimen (50 mg/kg, i.p.), we have shown that L-NAME significantly reduces NADPH-d activity in the SON of hyperosmotic late adulthood animals. Stimulated NADPH-d staining was similarly reduced in PCB and L-NAME-treated rats, suggesting that PCBs might act through NOS blockade to reduce osmotically-induced activation of the SON. This is consistent with results of an *in vitro* study showing that long-term exposure to individual PCB congeners, that are present in relatively high weight percent in the Aroclor 1254 mixture (PCB52, PCB138), reduces the activation of NO synthase and the whole glutamate-NO-cGMP pathway in response to activation of N-methyl-D-aspartate receptors (Llansola et al., 2010). These effects have been linked to reduced learning ability *in vivo*. In our study, the PCB-induced effects on NADPH-d in the SON of older animals occurred in tandem with significantly higher plasma osmolality during osmotic activation at 3–4 h post-stimulation indicating neuroendocrine disruption by ontogenic Aroclor 1254 exposure. In contrast, late adult hyperosmotic rats treated with L-NAME did not show significantly increased plasma osmolality, suggesting that *acute* pharmacological disruption of NOS (*via* L-NAME) was not as effective in comparison. Aroclor 1254 exposure produces effects on multiple key intracellular processes, in part, due to the combined actions of multiple PCB congeners (Kodavanti and Ward, 2005). Indeed, congeners like PCB153 present in Aroclor 1254, may not affect NADPH-d activity of NOS but still interfere with the content of the associated Ca^{2+}-binding protein calmodulin, a required protein for calcium-dependent as well as NO-dependent signaling (Llansola et al., 2009).

Central NO signaling appears to be a common target of environmental toxicants (Currás-Collazo, 2011). Industrially released heavy metals such as lead, manganese, and mercury have also been shown to alter central nitrergic systems after prenatal or postnatal exposure (Chetty et al., 2001; Freire et al., 2007). Evidence also exists for NO involvement in the neurotoxic actions of organohalogens such as polybrominated diphenyl ethers (PBDEs). NO functions in both normal and pathological CNS processes associated with endocrine, homeostatic as well as synaptic functions (Dawson and Dawson, 1996; Garthwaite and Boulton, 1995; Stern and Zhang, 2005). There is evidence that most of the dual actions of NO may be targets of PCBs. For example, short-term exposure to Aroclor 1254 exposure *in vitro* increases NO signaling and its downstream effectors, cGMP/PKG, in neuroblastoma cells, leading to cell death (Canzoniero et al., 2006). On the other hand, dopaminergic cell death has been linked to inhibition of NO signaling by PCBs (Kang et al., 2002; Yun et al., 2005), suggesting that dysregulation of NO signaling, either in the positive or negative direction, can have marked effects on cell survival.

In conclusion, *in utero* exposure to Aroclor 1254 produces abnormally reduced nitrergic expression in postnatal rats. Upon maturation exposed rats also show reduced stimulated NADPH-d responses, an effect not seen in rats exposed in adulthood. This effect persists into late adulthood, when the reduced NOS activity is coincident with significantly elevated plasma osmolality in response to hyperosmotic stimulation. NOS in the hypothalamus is affected by certain ortho- and hydroxy-substituted PCB congeners, to a relatively greater extent than are those of the cerebellum and hippocampus (Sharma and Kodavanti, 2002). Since NO has a central role in hypothalamic circuits governing cardiovascular and

osmoregulation, thyroid status, circadian rhythm, nutrient homeostasis, reproduction and growth (Canabal et al., 2007; Ding et al., 1994; Liu et al., 1998; Rettori et al., 1993), Aroclor 1254-induced disruption of NOS activity may represent one mechanism through which PCBs and similarly acting environmental toxicants may affect important regulatory physiological functions governed by the hypothalamus. These functions may include osmoregulatory capacity and blood pressure responses to stress in late adult rats which our lab has shown are significantly impaired by developmental exposure to similar organohalogens (Shah et al., 2011).

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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