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Prenatal carbon monoxide impairs migration of interneurons into the cerebral cortex

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ABSTRACT

Prenatal exposure to carbon monoxide (CO) disrupts brain development, however little is known about effects on neocortical maturation. We exposed pregnant mice to CO from embryonic day 7 (E7) until birth. To study the effect of CO on neuronal migration into the neocortex we injected BrdU during corticogenesis and observed misplaced BrdU+ cells. The majority of cells not in their proper layer colocalized with GAD65/67, suggesting impairment of interneuron migration; interneuron subtypes were also affected. We subsequently followed interneuron migration from E15 organotypic cultures of mouse neocortex exposed to CO; the leading process length of migrating neurons diminished. To examine an underlying mechanism, we assessed the effects of CO on the cellular cascade mediating the cytoskeletal protein vasodilator-stimulated phosphoprotein (VASP). CO exposure resulted in decreased cGMP and in a downstream target, phosphorylated VASP. Organotypic cultures grown in the presence of the phosphodiesterase inhibitor IBMX resulted in a recovery of the leading processes. These data support the idea that CO acts as a signaling molecule and impairs function and neuronal migration by acting through the CO/NO–cGMP pathway. In addition, treated mice demonstrated functional impairment in behavioral tests.

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1. Introduction

The prevalence of maternal smoking remains surprisingly high, ranging from 10 to 50% of pregnant women across different countries and socioeconomic groups (Toro et al., 2008). Prenatal exposure to cigarette smoke associates with a number of adverse clinical effects such as decreased weight and head circumference at birth (Kallen, 2000; Ward et al., 2007), as well as adverse neurological outcomes including increased risk for Sudden Infant Death Syndrome (Pinho et al., 2008) and attention deficit disorder (Milberger et al., 1998). The negative effects of maternal smoking are generally attributed to two major components of cigarette smoke: nicotine and carbon monoxide (CO). While substantial research demonstrates the biological consequences of maternal smoking, the vast majority of studies focus on the effects of nicotine, and often overlook the effects of CO.

Exposure to CO *in utero* causes profound effects in multiple regions of the developing brain, including the basal ganglia, cerebellum, hippocampus, cerebral cortex, and developing white matter (Ginsberg and Myers, 1976; Daughtrey and Norton, 1982; Mereu et al., 2000; Weiss et al., 2004; Benagiano et al., 2005). These effects are attributed to the ability of CO to produce hypoxia at low concentrations. CO also acts as a gaseous signaling molecule similar to nitric oxide (NO). At elevated intracellular levels, CO binds to all heme containing molecules including mitochondrial cytochromes, cytochrome P450, neuroglobin, and cytoglobin (Caughey, 1970; Sawai et al., 2005; Fago et al., 2006). The signaling role for CO first emerged from observations in smooth muscle cells indicating that CO stimulates soluble guanylate cyclase (sGC) to produce cGMP (Furchgott and Jothianandan, 1991). cGMP is an important second messenger for a number of developmental processes including neural plate responsiveness to Sonic hedgehog signaling (Robertson et al., 2001), neuronal growth cone extension, and responsiveness to semaphorin 3A (Song et al., 1998; Van Wagenen and Rehder, 2001). cGMP also activates cGMP dependent kinases, which phosphorylate important cytoskeletal proteins involved in neuronal migration, such as the actin associated cytoskeletal complex Ena/Vasodilator-stimulated phosphoprotein

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(VASP) (Goh et al., 2002). These processes are essential for brain development. Few have examined the direct effects of CO as a signaling molecule and its role in neuronal migration, a critical component of neocortical development (Knipp and Bicker, 2009). Therefore, this study examined the effects of prenatal CO exposure on the developing neocortex and characterized the structural and functional consequences.

Neocortical development involves the proliferation of neural stem cells followed by migration, and differentiation of neurons in a precisely regulated process. In mice, and mammals in general, the timing of this process is well characterized. Exposure to environmental toxins such as cigarette smoke (Gospe et al., 1996), cocaine (Crandall et al., 2004; Lee et al., 2011; McCarthy et al., 2011), alcohol (Kumada et al., 2007), bisphenol (Nakamura et al., 2007), methyl mercury (Choi et al., 1978), radiation (Algan and Rakic, 1997), methylazoxy methanol (Poluch et al., 2008) or ultrasound (Ang et al., 2006) can result in a myriad of cortical defects based on abnormal proliferation and migration, aberrant cell death patterns, or abnormal gliogenesis (Kriegstein, 1996; Krauss et al., 2003; Pang et al., 2008; McCarthy et al., 2011).

Although the results of prenatal CO exposure have been studied in the brain, the specific effects on neocortical development and neuronal migration have not. Here we report that prenatal exposure to levels of CO similar to that seen in heavy smokers results in displaced cells born during early and late neurogenesis (E12 or E16) in the mouse. Furthermore, CO exposure causes alterations in the distribution of GABAergic interneurons in the neocortex. A subpopulation of interneurons expressing parvalbumin was also susceptible to this insult. We propose the mechanism mediating this effect is in part due to impaired VASP phosphorylation *via* partial inhibition of the CO/NO–sGC–cGMP pathway. CO exposure also results in behavioral changes in mice consistent with disrupted neocortical development.

2. Materials and methods

2.1. Animals and exposures

All animal work was approved by the USU IACUC. Timed pregnant mice (CD-1) were purchased from Charles River Laboratories (Wilmington, MA). Pregnant dams were housed individually in their cages within clear acrylic environmental chambers approximately 24″ × 24″ × 21″. Animals were provided with nestlets, food and water *ad libitum*. The chambers of all groups received an air flow of approximately 20 L/min. A CO concentration of 150 ppm was maintained in one chamber by mixing 7.5% CO with the carrier air flow and monitoring at least once per day (Interscan RM14-500M, Chatsworth, CA). This amount generates HbCO (carboxyhemoglobin) levels in the blood similar to those in a heavy smoker (10–16%). Smokers consume cigarettes to maintain a constant level of plasma nicotine (Henninfield and Goldberg, 1988). An average daily CO concentration for a typical experiment was 148 ± 3.8 (mean ± SEM) ppm. Dams displayed no behavioral changes during the exposure period and there was no mortality. See Supplemental Table 1 for effects of maternal CO exposure.

Our goal was to achieve the CO concentration of a person smoking one–two packs of cigarettes a day. Before finalizing on 150 ppm we attempted concentrations of 75 ppm and 500 ppm. We found that the lower concentration was not effective in achieving the required blood levels of HbCO (as seen in Supplemental Table 1). The higher concentration, however, produced behavioral changes in the pregnant mice that indicated toxicity. We therefore conducted our study using 150 ppm, which produced the desired blood levels and did not affect behavior, weight gain or rate of weight gain of the dams.

For *in vitro* experiments, slices were maintained in an incubator (95% O_2, 5% CO_2, 37 °C) at either 0 or 50 ppm CO. Exposure to 150 ppm was attempted in the organotypic slices, but at this exposure the cultures were not viable. Therefore, we reduced the concentration to 50 ppm. Migration was viable at 50 ppm and slices survived for the duration of the experiment. CO levels in the incubator were monitored with a GasBadge Pro CO detector (Industrial Scientific, Frederick, MD).

2.2. Histology and laminar measurements

For histologic and immunohistochemical experiments, mice were anesthetized using sodium pentobarbital solution (50 mg/kg), perfused with ice-cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were removed and stored in 4% paraformaldehyde at 4 °C; they were then sectioned, mounted, stained, and imaged within 72 h. We examined the somatosensory cortex in pups exposed to CO *in utero* at three ages: postnatal day 4 (P4), 11 (P11), or adult (P56 or later). 40 μm thick coronal sections from somatosensory cortex were stained with cresyl violet as previously described (Gittins and Harrison, 2004). At each age one pup from 4 different litters was chosen and 3 adjacent sections were evaluated. Images were taken using 10× magnification (Zeiss Axiovert 200, Thornwood, NY). The cortical thickness was measured as the distance from the pia to the white matter; the thickness of layers 1, 2–4, 5 and 6 was determined by cell morphology and density and measured using Axiovision software. Neuronal distributions and laminar measurements data were analyzed using a two-way ANOVA followed by a Holm–Sidak *post hoc* test for multiple comparisons (*e.g.*, Poluch et al., 2008).

2.3. Bromodeoxyuridine (BrdU) birthdating and Immunohistochemistry

For birth dating studies, 20 pregnant dams were injected 3× i.p. at 45 min intervals with bromodeoxyuridine (BrdU; 50 mg/kg, Sigma, St Louis MO) diluted in sterile saline on E12 or E16 as described previously (Miller and Nowakowski, 1988; Noctor et al., 1997; Wojtowicz and Kee, 2006; Duque and Rakic, 2011). Three injections were given due to the relatively short half-life of the compound and to maximize the quantity of labeled neurons born in a specific time period. Alternative groups of mice that did not receive BrdU were subjected to behavioral testing as described below. In all groups, animals were examined postnatally as adults (P56 or later). 40 μm thick vibratome prepared coronal sections were incubated overnight at 4 °C in either rabbit IgG antibodies against GAD 65/67 (1:500, Sigma), MAP2 (1:500, Sigma), CAMKIIα (1:200, Chemicon) or a mouse IgG antibody against parvalbumin diluted in buffer containing PBS, normal goat serum (NGS, 10%), and Triton X100 (0.3%). The slices were washed in PBS and incubated with appropriate secondary antibodies (Alexa Fluor 546-conjugated goat anti-rabbit or goat anti-mouse, 1:500). Slices containing somatosensory cortex were subsequently processed for BrdU immunohistochemistry as previously described (Poluch et al., 2008). Slices were incubated in ethanol (70%) for 10 min, then placed in 2 N HCl at 37 °C for 1 h, and rinsed in 0.1 borate buffer, pH 8.5 for 20 min. After washing in PBS, slices were incubated overnight at 4 °C in polyclonal rat IgG antibodies against BrdU (1:200; Accurate Chemical and Scientific Corp. Westbury, NY) in buffer containing PBS, NGS (10%), and Triton X-100 (0.3%). Slices were then washed in PBS, and then incubated for 3 h at room temperature with Alexa Fluor 488-conjugated goat anti-rat IgG (1:500). Slices were mounted onto slides using Mowiol, coded to a blind observer as to the treatment group, and coverslipped overnight prior to imaging. For analysis of each antibody or BrdU