Neuroscience Letters 452 (2009) 75–78

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Prenatal ozone exposure abolishes stress activation of Fos and tyrosine hydroxylase in the nucleus tractus solitarius of adult rat

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ARTICLE INFO

Article history:
Received 21 July 2008
Received in revised form
27 November 2008
Accepted 1 January 2009

Keywords:
Pregnant rats
Oxidative stress
Immobilization stress
Air pollution
Immunohistochemistry
Confocal microscope

ABSTRACT

Ozone (O_3) is widely distributed in the environment, with high levels of air pollution. However, very few studies have documented the effects on postnatal development of O_3 during pregnancy. The long-term effects of prenatal O_3 exposure in rats (0.5 ppm 12 h/day from embryonic day E5 to E20) were evaluated in the adult nucleus tractus solitarius (NTS) regulating respiratory control. Neuronal response was assessed by Fos protein immunolabeling (Fos-IR), and catecholaminergic neuron involvement by tyrosine hydroxylase (TH) labeling (TH-IR). Adult offspring were analyzed at baseline and following immobilization stress (one hour, plus two hours' recovery); immunolabeling was observed by confocal microscopy. Prenatal O_3 increased the baseline TH gray level per cell ($p < 0.001$). In contrast, the number of Fos-IR cells, Fos-IR/TH-IR colabeled cells and proportion of TH double-labeled with Fos remained unchanged. After stress, the TH gray level ($p < 0.001$), number of Fos-IR cells ($p < 0.001$) and of colabeled Fos-IR/TH-IR cells ($p < 0.05$) and percentage of colabeled Fos-IR/TH-IR neurons against TH-IR cells ($p < 0.05$) increased in the control group. In prenatal-O_3 rats, immobilization stress abolished these increases and reduced the TH gray level ($p < 0.05$), indicating that prenatal O_3 led to loss of adult NTS reactivity to stress. We conclude that long-lasting sequelae were detected in the offspring beyond the prenatal O_3 exposure. Prenatal O_3 left a print on the NTS, revealed by stress. Disruption of neuronal plasticity to new challenge might be suggested.

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Ozone (O_3) is a major environmental pollutant and exposure to O_3 has become more and more frequent in and around urban areas. Most reports on the effects of O_3 have focused on the respiratory tract, lung and respiratory function and airway inflammation in adults. However, there is now accumulating evidence that O_3 induces extrapulmonary effects, particularly on the central nervous system, resulting in disruption of sleep patterns [20], alteration of social behavior [17], memory deficit [22], impaired respiratory control [4]. These neurobehavioral changes have been linked to changes in dendritic spine density and in the metabolism of brain neurotransmitters including catecholamines [5,9,29] and serotonin [20], and to oxidative stress [22]. Thus, the physiological, neurochemical and morphological sequelae reveal the plasticity of the central nervous system in response to O_3 exposure. Moreover, by affecting kindling development [7], O_3 disrupted morphofunctional changes underlying the hyperexcitability reported in this model of epilepsy. Likewise, O_3 modified the ventilatory response to hypoxia [28], suggesting that O_3 may interfere with mechanisms involved in the response to hypoxia. These reported data indicated that O_3 exposure in adults may induce firstly, morphofunctional changes, i.e. plastic changes in the central nervous system at baseline and secondly, interferences with mechanisms involved in response to stress.

The effects of O_3 are age-dependent [12,28], and during pregnancy susceptibility to oxidants is increased [11]. However, very little is known about the effects of O_3 exposure during pregnancy on the postnatal development of offspring. Some reports indicate that 1 ppm prenatal O_3 altered the morphology [23,24], neurotransmitter content [8], and total area and number of Purkinje cells [24] in the cerebellum. Moreover, 0.3–0.6 ppm prenatal O_3 reduced nerve growth factor levels in the hippocampus and increased brain-derived neurotrophic factor content in the striatum [27]. Taken together, these data suggest that prenatal O_3 exposure affects the development of the central areas of offspring.

The present study in rats sought to determine whether prenatal O_3 exposure (0.5 ppm, 12 h/day from embryonic day 5 to embryonic day 20) induces sequelae in the nucleus tractus solitarius (NTS) of offspring in adulthood. The NTS, part of the dorsal medulla, was selected as being a signaling pathway for respiratory control [2], containing clusters of noradrenergic neurons regulating

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doi:10.1016/j.neulet.2009.01.027

breathing through connections to the adjacent dorsal respiratory group, and as being a central area that is reactive to O_3 exposure by catecholamine biosynthesis and respiratory change [4,5], thus revealing neuroplasticity. The neuronal response to prenatal O_3 was assessed by Fos protein immunoreactivity (Fos-IR), and the involvement of the catecholaminergic neurons by the immunoreactivity of tyrosine hydroxylase (TH) (TH-IR), the rate-limiting enzyme of catecholamine biosynthesis. In addition, given that stress might reveal abnormalities undetected at baseline [21], animals were subjected to immobilization stress for 1 h, Fos [18] and TH proteins being detected 2 h later.

Male and female Sprague-Dawley rats (IFFA Credo, France) were mated at night and the females presenting sperm-positive smears were defined as being at embryonic day 0 (E0). The pregnant rats (300–320 g) were then housed in an air-conditioned room at $26 \pm 1\,^{\circ}C$ with a 12 h light-dark cycle. During the entire experimental procedure, animals were allowed free access to food (UAR AO3, 3.2 kcal/g) and water. Pregnant rats were randomly assigned to the prenatal-O_3 or control group. Experiments were carried out according to the ethical principles laid down by the French Ministry of Agriculture and EU Council Directives for the care and use of laboratory animals (No 02889).

On embryonic day 4 (E4), the pregnant females ($n = 4$) were placed in a plexiglass chamber and exposed daily from E5 to E20 to 0.5 ppm O_3 during their activity period, from 7 pm to 7 am, according to the protocol previously described [28]. Although this level of O_3 is about twice as high as that measured in polluted urban areas, the rat respiratory system is less sensitive to O_3 than that of humans and a concentration of at least 0.5 ppm is required to mimic the pathological effects on the human lung [13]. As previously described [5,28,29], the plexiglass chamber was under constant air flow; filtered air was passed across an ultraviolet light source so as to obtain an O_3 concentration of 0.5 ppm. The concentration of O_3 was monitored on a calibrated UV photometric O_3 monitor (ref 41 M, Environment SA, Poissy, France; range ± 0.0001 ppm) connected to the outlet line of the chamber. The control pregnant females ($n = 4$) were simultaneously kept in a chamber of the same size provided with filtered ambient air at the same flow rate. At E20, the two groups of pregnant rats were housed individually and birth occurred in normoxia.

At birth, prenatal-O_3 and control pups were redistributed to nursing females never exposed to O_3, in order to rule out possible delayed O_3 exposure effects on the dam. Prenatal-O_3 pups and control pups were randomized within treatment groups and litters were randomly culled to 10 pups (eight males, two females) to ensure standard nutrition. Pups were housed with their nurse in plastic breeding cages until study.

At adulthood, the control and prenatal-O_3 offspring were divided in four groups: four male control rats (403.8 ± 11.1 g) were left undisturbed in their home cage, to constitute the baseline control group; four male control rats (406.5 ± 6.2 g) were subjected to immobilization stress, to constitute the stress control group; four male prenatal-O_3 rats (413.5 ± 3.5 g) were left undisturbed in their home cage, to constitute the baseline prenatal-O_3 group; and four male prenatal-O_3 rats (402.5 ± 22.5 g) were subjected to immobilization stress, to constitute the stress prenatal-O_3 group. For the immobilization stress, rats were restrained individually in a transparent plastic cylinder (7 cm in diameter and 19 cm long) for 1 h and then allowed to recover in their respective breeding cages for 2 h. The minimal number of offspring to achieve statistical significance was used, and special attention was paid to minimize animal suffering.

Animals were anesthetized (pentobarbital, 0.1 ml/100 g bodyweight i.p.) for sacrifice and fixed by transcardiac perfusion of phosphate buffer (0.1 M, pH 7.4) containing 4% paraformaldehyde. Brains were dissected out and postfixed in the same fixative overnight. Then they were cryoprotected, frozen and stored at $-80\,^{\circ}C$. Twenty-five micrometers thick coronal sections were collected from the medulla oblongata (from -13.80 to -14.60 mm rostro-caudally to Bregma [19]) by cryostat (Reichert-Jung, Germany).

The fixed brain slices were incubated overnight with rabbit polyclonal c-Fos antibody (Oncogene 2b-5, dilution 1/1000) and mouse monoclonal TH antibody (dilution 1/2000) in phosphate saline buffer (0.1 M and 0.9% NaCl, PBS) containing 0.05% sodium azide, 0.1% bovine serum albumin and 0.3% Triton (PBS-T-Az). Primary antibodies were incubated for 45 min with Alexa 488 conjugated affinity-purified goat anti-rabbit (dilution 1/300) and Alexa 633 conjugated affinity-purified goat anti-mouse (dilution 1/200; Jackson Laboratories Inc., PA, USA), in PBS containing 0.5% gelatin. After antibody incubation, all washes used PBS-T-Az. Slices were counterstained with 2 μg/ml Hoescht. Series of five slices (1 μm thick) were observed under a Leica confocal system TCS-SP2 (63× magnification) and captured with a filter set at 505–559 nm for Alexa 488 (c-Fos protein), 678–768 for Alexa 633 (TH protein) and 403–496 nm (Hoechst). Sources were analyzed and merged using Adobe Photoshop and JASC or Image J.

Statistical analyses were performed with Prism software using two-way ANOVA and Newman–Keuls correction or one tailed *t*-test. Data were expressed as mean ± SEM. The significance threshold was set at $P < 0.05$.

In the four groups, immunocytochemical analysis detected TH-IR and Fos-IR cells in the NTS. TH immunoreactivity was cytoplasmic (Fig. 1A). Fos immunoreactivity was restricted to the nucleus (Fig. 1B) and some neurons were colabeled TH-IR/Fos-IR (Fig. 1C).

At baseline, prenatal O_3 reduced the total nucleus count (123.7 ± 2.2 nuclei per section in baseline prenatal-O_3 rats *vs.* 132.6 ± 2.5 in baseline controls). As reported in Table 1, the TH gray level per cell was significantly higher in the baseline prenatal-O_3 group than in baseline controls. In contrast, prenatal O_3 exposure failed to alter: (a) the number of TH neurons (7.4 ± 0.5 per section in baseline prenatal-O_3 rats *vs.* 6.5 ± 0.5 in baseline controls), (b) TH arborization length (218 ± 10 pixels in baseline prenatal-O_3 rats *vs.* 218 ± 10.5 pixels in baseline controls), (c) the number of Fos-IR cells, (d) the number of colabeled Fos-IR/TH-IR cells, or (e) the percentage of colabeled Fos-IR/TH-IR neurons against TH-IR cells (Table 1).

In control rats, immobilization stress increased (a) the TH gray level per cell, (b) the number of TH–IR neurons, (c) the number of Fos-IR cells, (d) the number of colabeled Fos-IR/TH-IR cells, and (e) the percentage of colabeled Fos-IR/TH-IR neurons against TH-IR cells. In contrast, in the prenatal-O_3 group, immobilization stress induced no change in Fos and TH patterns except for a slight decrease in TH gray level per cell. Consequently, after immobilization stress, the TH gray level per cell and number of Fos-IR cells per section in the NTS were lower in the prenatal-O_3 stress group than in stress controls (Table 1).

The immunocytochemical results show that prenatal O_3 exposure had an impact on the NTS of adult rats, inducing long-term sequelae revealed under stress; it disrupts the pattern of immobilization stress inducing elevated TH and Fos levels.

The present study confirms that the NTS of adult control rats is immunoreactive to Fos and contains few cells labeled with Fos compared with TH-IR neurons (15–20%), as reported previously [1,14]. Prenatal O_3 exposure decreased the total number of nuclei, suggesting long-term cell death, as reported in the cerebellum of prenatal-O_3 rats [24] and in fetal paraventricular nuclei after prenatal stress [30]. At baseline, prenatal O_3 increased TH gray level per cell. However, other forms of prenatal stress such as hypoxia were reported to have no obvious effect on adult NTS TH activity [22,21]. These findings suggest that TH protein expression may be dependent on the type of prenatal environmental stress. Prenatal

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