

# Long-Term Prenatal Hypoxia Alters Maturation of Adrenal Medulla in Rat

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## ABSTRACT

Catecholamine release from the adrenal medulla glands plays a vital role in postnatal adaptation. A number of pathologic situations are characterized by oxygen deficiency. The objective of the present study was to determine the influence of long-term prenatal hypoxia on maturation of the adrenal medulla. Pregnant rats were subjected to hypoxia (10% O<sub>2</sub>) from the fifth to the 20th d of gestation. The offspring were examined on the 19th d of gestation (E19), the day of birth (P0), and at postnatal (P) day of life P3, P7, P14, P21, and P68. The catecholamine content and activity of tyrosine hydroxylase (TH) *in vivo* were assayed by HPLC with electrochemical detection. Cellular expression of TH and phenylethanolamine *N*-methyl transferase was evaluated by protein immunohistochemistry and *in situ* hybridization of the corresponding mRNA species. Exposure to prenatal hypoxia reduced the epinephrine content of the adrenal medulla on E19, P0, P3, and P7 while increasing the norepinephrine content on E19, P0, and P14. Furthermore, the peak epinephrine to norepinephrine ratio appearing between P7 and P10 in the normoxic offspring was absent in the hypoxic offspring. The *in vivo* TH activity was increased on P3 and P14 and decreased on P68. The percentage of chromaffin cells in the medulla expressing TH and phenylethanolamine *N*-methyl transferase was lowered on E19, P0, and P7. TH and phenylethanolamine *N*-methyl transferase mRNA levels were reduced on P7. Clearly prenatal hypoxia

results in major changes in adrenal catecholamine stores and synthesis during the perinatal period, which persist into adulthood. The capacity to cope with postnatal stress might be disturbed as a consequence of prenatal hypoxia. (*Pediatr Res* 51: 207–214, 2002)

### Abbreviations

**DOPA**, L-3,4-dihydroxyphenylalanine  
**E**, epinephrine  
**E19**, d 19 of embryogenesis  
**HPLC-ED**, HPLC with electrochemical detection  
**Hx**, hypoxic  
**NE**, norepinephrine  
**Nx**, normoxic  
**PNMT**, phenylethanolamine *N*-methyl transferase  
**P0**, day of birth  
**P3**, d 3 of postnatal life  
**P7**, d 7 of postnatal life  
**P14**, d 14 of postnatal life  
**P21**, d 21 of postnatal life  
**P68**, d 68 of postnatal life  
**TH**, tyrosine hydroxylase  
**11  $\beta$ -OHSD**, 11 $\beta$ -hydroxysteroid dehydrogenase

The release of catecholamines plays an essential role in initial adaptation to extrauterine life, particularly in connection with perinatal asphyxia (1). Circulating catecholamines originate to a large extent from the adrenal medulla glands and serve vital functions in relation with cardiovascular, respira-

tory, and metabolic responses to the stress associated with birth (1–4). In the rat, the chromaffin cells of the adrenal medulla are still immature at the time of birth and undergo extensive biochemical, morphologic, and functional changes during postnatal development.

Catecholamine synthesis is catalyzed by cytoplasmic enzymes, including TH, which represents the rate-limiting step, and PNMT, which catalyzes the biosynthesis of E. At birth, increases in both the plasma and adrenal levels of catecholamines occur (5, 6). Marked increases in the levels of TH mRNA and of dopamine  $\beta$ -hydroxylase mRNA are observed in the adrenal, thus reflecting the surge in catecholamines at the

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time of birth. In contrast, the increase in PNMT mRNA is delayed (7).

The volume of the adrenal medulla (containing chromaffin cells, neuronal tissue, and interstitial tissue) changes with age. Thus, at birth, the chromaffin cells constitute only 34% of the adrenal medulla gland volume, and this value increases to 65% in the adult animals (8). At birth, each chromaffin cell exhibits granules containing both E and NE. However, after 4 d of postnatal life, the chromaffin cells begin to differentiate into two distinct populations, *i.e.* the E cells, expressing both TH and PNMT, and the NE cells, expressing only TH (9, 10).

Functionally, the adrenal medulla demonstrates the capacity to release catecholamines directly (*i.e.* without neural stimulation) during the first 10 d after birth (11–13). Thereafter, secretion from the adrenal medulla is stimulated by the splanchnic nerve.

The biochemical, morphologic, and functional immaturity of rat adrenals during postnatal development might render these glands more sensitive to environmental stress, which could alter the process of maturation. A number of pathologic situations (including maternal anemia; reduced placental blood flow as a consequence of maternal hypertension or smoking; ethanol consumption by the mother; reduced placental size, or reduced inhalation of oxygen by the mother at high altitude; recurrent apnea of prematurity; obstructive sleep apnea; and bronchopulmonary disease) are known to produce environmental stress in the fetus or neonate. All of these situations lower the availability of oxygen to fetal or neonatal cells.

We have reported previously that early postnatal exposure to Hx conditions alters the pattern of maturation of the sympathetic system, including the adrenal glands (14). Short-term prenatal exposure to Hx retards intrauterine growth, an effect that remains until the 10th d of postnatal life (15, 16). Furthermore, such prenatal Hx is associated with an increased risk for neurologic abnormalities during development (17), including cognitive and behavioral disorders (18–21). Holgert *et al.* (22) have found that short-term Hx (11–13% O<sub>2</sub>) during the last 3 d of gestation induces an increase in the level of TH mRNA in the fetal adrenal gland 1 d before birth. However, there are presently no studies available dealing with the effects of long-term Hx on the maturation of the adrenal medulla.

Our working hypothesis was that prenatal Hx alters the maturation of the rat adrenal gland. Thus, the goal of the current study was to characterize the influence of long-term prenatal Hx in rats (10% O<sub>2</sub>) lasting from the fifth to 20th d of gestation, *i.e.* for 2 wk, on catecholamine biosynthesis in the adrenal medulla from 2 d before birth through to adulthood. First, we focused on the ontogenesis of the contents of NE and E and of *in vivo* TH activity. Subsequently, we examined the maturation of the chromaffin cells, using both immunocytochemical detection of the TH and PNMT proteins and *in situ* hybridization of the corresponding mRNA species.

## METHODS

### Animals and Experimental Procedures

Sixty pregnant Sprague-Dawley rats (IFFA-CREDO, l'Arbresle, France) were housed in a temperature-controlled

room (26 ± 1°C) with a 12-h light/dark cycle and allowed free access to food and water. The first day of gestation was determined on the basis of a vaginal smear. Pregnant rats were first exposed to Hx on the fifth day of gestation so as not to disturb implantation of the embryo.

From the fifth to 20th d of gestation, the experimental group was housed in a Plexiglas chamber, with a normobaric Hx atmosphere consisting of 10% O<sub>2</sub>/90% N<sub>2</sub>. The O<sub>2</sub> and CO<sub>2</sub> contents of this atmosphere were monitored twice daily using Servomex analyzers (Servomex Co., Norwood, MA, U.S.A.). The CO<sub>2</sub> expired by the rats was eliminated by circulating the atmosphere through soda lime, so that the atmospheric content of this gas never exceeded 0.1%. The water contained in the expired gas was trapped in a chilled glass tank.

On the 20th d of gestation, Hx pregnant rats were returned to Nx conditions (21% O<sub>2</sub>), under which they gave birth. Immediately after birth, the male pups were grouped together, and then randomly redistributed to nursing dams never exposed to Hx conditions (10–12 pups per dam). This procedure was followed to avoid sex differences, eliminate variations between litters, ensure a standard nutritional status, and avoid any possible effect of Hx on the nutritional quality of the milk. From each litter one pup was chosen for further analyses. These offspring were designated as the prenatal Hx group. Pups from another 60 pregnant Sprague-Dawley rats, whose gestation occurred under Nx conditions (21% O<sub>2</sub>), but were otherwise treated in the same manner, were designated as the Nx control group.

A total of 273 prenatal Hx and Nx control offspring were examined on E19, at birth (*i.e.* 2–4 h after birth), and on P3, P7, P14, P21, and P68. The pups were killed by cooling at P0 and P3, and by cervical dislocation at P7, P14, P21, and P68. The experiments were performed according to the ethical principles formulated in the French (Ministère de l'Agriculture) and EU Council Directives for the care of laboratory animals.

One hundred fifty-three pups were used for measurement of NE and E levels, immunohistochemistry, and *in situ* hybridization. In the remaining 120 animals, *in vivo* TH was assayed.

### Neurochemical Analyses

These analyses were performed using HPLC-ED.

**Determination of catecholamine levels.** After sacrifice, the pup's right adrenal was rapidly dissected out, weighed, and placed in 100  $\mu$ L of 0.4 M perchloric acid containing 2.7 mM EDTA-Na<sub>2</sub>, and then this suspension was diluted to 200  $\mu$ L with a solution containing dihydrobenzylamine as an internal standard. Adrenal glands were subsequently disrupted by ultrasonication, and the resulting homogenates were centrifuged (8800  $\times$  g, 10 min.). Thereafter, the supernatant was diluted in 0.02 M acetic acid, and a 5- $\mu$ L aliquot was injected into a reverse-phase column (Licrospher RP 18 ec, 5  $\mu$ m, 250  $\times$  3 mm). Elution was performed with a mobile phase consisting of 50 mM citric acid, 50 mM sodium acetate, 1 mM EDTA-Na<sub>2</sub>, 437 mM acetic acid, 2.13 mM heptane sulfonate, and 7% methanol at a flow rate of 0.4 mL/min. NE and E were quantified at +0.67 V with an Ag<sup>+</sup>/AgCl electrode.

**Estimation of TH in vivo.** TH *in vivo* in the adrenal gland was measured as the accumulation of DOPA during a period of 20 min after intraperitoneal injection of m-hydroxybenzylhydrazine (NSD-1015, 100 mg/kg; Sigma Chemical Co., St. Louis, MO, U.S.A.), an inhibitor of DOPA decarboxylase. DOPA accumulation under these conditions is considered to be a good indicator of TH activity (23, 24).

The right adrenal glands of pups killed on P0, P3, P7, and P14 were treated as described above, with the exception that  $\alpha$ -methyl DOPA was used as internal standard. The low levels of NE and E in these samples did not interfere with the DOPA assay. Therefore, aliquots (5  $\mu$ L) of these supernatants were injected directly onto the HPLC for DOPA analysis, without prior purification.

In the case of pups killed from P21 to adulthood, the high levels of adrenal E and NE interfered with determination of the low levels of DOPA. This problem was eliminated by removing the catecholamines from the samples before the HPLC-ED procedure. Following the protocol described by Hayashi *et al.* (23), catecholamines were trapped on a cation-exchange column (Bio-Rex 70), to which DOPA and the internal standard ( $\alpha$ -methyl DOPA) did not bind. Briefly, the right adrenal was homogenized in 1 mL of 0.1 M perchloric acid containing 2.7 mM EDTA- $\text{Na}_2$  and 1 mg/mL sodium bisulfite, along with the internal standard  $\alpha$ -methyl DOPA. After centrifugation (8800  $\times$  g, 15 min), the pH of the supernatant (0.9 mL) was adjusted to be between 6 and 7 using 1 M  $\text{K}_2\text{CO}_3$ . After cooling in an ice bath for 20 min, the precipitated  $\text{KClO}_4$  was removed by centrifugation (3000  $\times$  g, 10 min) and the supernatant (850  $\mu$ L) applied to a column (7 mm  $\times$  19 mm) of Bio-Rex 70. The effluent and the following 3.5-mL fraction eluted with ice-cooled-water were collected for subsequent determination of DOPA. This eluate was then purified by alumina extraction as follows: the pH of the solution was adjusted to 8.4–8.6 with 1 M Tris-HCl buffer. Thereafter, the mixture was shaken for 20 min with 20 mg of acid-washed alumina, the supernatant discarded, and the alumina washed three times with 1 mL, each time with rinsing solution containing 14 mM Tris-HCl and 2.7 mM EDTA- $\text{Na}_2$ . DOPA and  $\alpha$ -methyl DOPA were then eluted from the alumina with 350  $\mu$ L of 0.3 M perchloric acid containing 1 mM EDTA and 1 mg/mL sodium bisulfite. This eluate was shaken (5 min) and centrifuged (8800  $\times$  g, 15 min), and an aliquot (10  $\mu$ L) was subsequently injected onto the HPLC-ED for DOPA assay.

Elution from the reverse-phase column (Spherisorb ODS-2, 5  $\mu$ m, 125  $\times$  4 mm) was accomplished using 50 mM citric acid, 50 mM sodium acetate, 1 mM EDTA- $\text{Na}_2$ , 437 mM acetic acid, 4.3 mM octane sulfonate, and 4% methanol at a flow rate of 0.9 mL/min. Eluted DOPA was quantified at +0.67 V via an  $\text{Ag}^+/\text{AgCl}$  electrode.

### Immunohistochemistry

Immunohistochemistry was performed on the left adrenal glands from six pups in the first group, killed at E19, P0, P7, and P21 ( $n = 44$ ), using the avidin-biotin peroxidase complex (25, 26). Briefly, the adrenals were first fixed by immersion in Bouin-Hollande solution for 24 h. After dehydration, these

samples were embedded in paraffin and sliced into adjacent sections (4  $\mu$ m thick). After removal of the paraffin, the sections were treated with hydrogen peroxide (3.75%) in methanol for 30 min, progressively rehydrated, and thereafter incubated with 5% bovine serum albumin in PBS (50 mM sodium phosphate, pH 7.45). Consecutive sections were incubated overnight in a moist chamber and then for 2 h at 37°C with the primary rabbit anti-TH (diluted 1/1500; Institut Jacques Boy, Reims, France) and anti-PNMT (diluted 1/1000; a gift from Dr. L. Denoroy) antibodies. The immunostaining was detected by incubation with the biotinylated secondary donkey anti-rabbit serum antibodies (diluted 1/200 and incubated for 45 min at 37°C; Amersham, Buckinghamshire, U.K.) and thereafter with the streptavidin-peroxidase complex (diluted 1/200 and incubated for 2 h at 37°C; Amersham). Peroxidase labeling was visualized by incubation in PBS (50 mM sodium phosphate, pH 7.45) containing diaminobenzidine (0.125%; Sigma Chemical Co., Saint Quentin, France) and ammonium nickel sulfate (0.08%).

At each age, adrenal sections were imaged and digitized using a tri-charge-coupled device color video camera (DXC 930P tri-CCD color, Sony Corp., Tokyo, Japan) mounted on a dissecting microscope (Diaplan, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) via a vario-TV 0.55–0.8 Photomount tube (Leica Imaging System Ltd, Cambridge, U.K.). Digitization produced a gray-scale image, from which positively stained surfaces were quantitated using a PC Leica Q500IW computer (Leica Imaging System Ltd) equipped with Q Win V2.0 software (Leica Imaging System Ltd). The total surface of the adrenal medulla was obtained by defining the boundary of the peripheral chromaffin cells. The surface of the adrenal medulla that was immunoreactive for TH (TH-ir<sup>+</sup>) and PNMT (PNMT-ir<sup>+</sup>) was determined using serial sections. The relative amounts of the surface occupied by TH-ir<sup>+</sup> cells and PNMT-ir<sup>+</sup> cells were obtained by dividing the positively staining surface by the total surface of the adrenal medulla.

### *In Situ* Hybridization

*In situ* hybridization was performed on the left adrenal glands from seven pups in the Nx control group and eight pups in the Hx group. All animals were 7 d old. For this purpose the adrenals were removed and frozen in liquid nitrogen. All adrenals from the Nx control group of pups were embedded together to get only one piece to section. Adrenal glands of the Hx group were also embedded all together. The two groups of glands were fully sliced into sections 12  $\mu$ m thick, and thawed onto the same set of slides (ProbeOn microscope slides, Fisher Scientific, Pittsburgh, PA, U.S.A.). Synthetic oligonucleotide probes complementary to the mRNAs encoding for rat TH (27) and PNMT (28) were used. These probes were labeled with  $\alpha^{35}\text{S}$ -dATP (PerkinElmer Life Science Products, Boston, MA, U.S.A.) at their 3'-ends using deoxynucleotidyl transferase (Amersham) and subsequently purified on Nensorb 20 columns (PerkinElmer Life Science Products).

Labeling with the probes and hybridization of the nonfixed tissue were carried out for 16 h at 42°C (29). The sections thus labeled were then exposed to x-ray film (Amersham) for 17 h

in the case of TH and 23 h for PNMT. Comparison of the relative levels of mRNA was accomplished by hybridizing adrenal sections from Nx control and prenatal Hx animals together on the same slide, *i.e.* using identical probe concentrations and hybridizing conditions. All TH hybridized slides (or PNMT) were exposed within one film to compare all slides together. Adrenal section images were captured and digitized using the same video camera as previously described (see "Immunohistochemistry"). The OD of the TH mRNA (or PNMT mRNA) of the adrenal medulla was semiquantified with the same computer and software as previously described. The specificity of the oligonucleotide labeling has been verified by adding the corresponding sense probe to the incubation cocktail.

### Statistical Analysis

The levels of NE and E were expressed as picomoles per gland. In comparison to the adrenal cortex region, the relative weight of the medulla varies at different stages of development. Accordingly, the total weight of the adrenal gland does not accurately reflect the weight of the medulla, so that these contents were not expressed per gland weight (5). The ratios between the E and NE contents were also calculated.

The *in vivo* TH activity was expressed as picomoles of DOPA accumulated per adrenal per 20 min (mean  $\pm$  SEM).

The immunohistochemical data were expressed as the percentage of the adrenal medulla surface that demonstrated positive immunoreactivity (mean  $\pm$  SEM), according to Tomlinson and Coupland (9).

The levels of TH mRNA and PNMT mRNA were expressed as ODs (mean  $\pm$  SEM).

Before statistical analysis by ANOVA, values demonstrating heterogeneous variance were transformed to the corresponding logarithmic values (30). At each age, the values for the Nx control and prenatal Hx groups were compared using one-way (prenatal treatment) ANOVA, followed by Fisher's protected least significant difference test. For each treatment during the development, the data were also analyzed using one-way (age) ANOVA, followed by Fisher's protected least significant difference test. A *p* value of  $<0.05$  was considered to be statistically significant.

## RESULTS

**Viability and growth.** Exposure of pregnant female rats to Hx from the fifth to the 20th d of gestation resulted in a

decrease in the mean litter size (from 13 pups to seven) and an increase in the mean weight of the placenta (from 0.560 to 0.677g; +21%) and in the mean ratio between placenta and fetal body weights at E19 (0.212 *versus* 0.304; +43.4%). Prenatal hypoxia also significantly reduced the body weight of the offspring at E19 (-23.4%), P0 (-31.2%), P3 (-8.9%), and P7 (-8.8%) while increasing this weight on P14 (+8%). During the following weeks (P21 and P68), the body weight of pups subjected to prenatal Hx was unaffected. Moreover, in these pups there was a decreased weight of the adrenals at birth (-37.3%) and an increased weight on P14 (+42.9%; Table 1).

**Neurochemical analyses.** In the Nx rats, the total adrenal content of catecholamines increased progressively from the end of the gestation until adulthood (Table 2).

Prenatal Hx reduced the adrenal catecholamine content before birth (-30.5% at E19), but this value increased at the time of birth (+25% on P0). The NE content was higher in rats subjected to prenatal Hx on E19 (+28%), at birth (+183%), and on P3 (+32%) and P7 (+67%). On the other hand, in these same rats, the adrenal E content was lower before birth and on P14 (-67% at E19, -14% on P0, and -4.5% on P14; Table 2).

In the case of the Nx control pups, the E/NE ratio increased from E19 ( $1.76 \pm 0.15$ ) until adulthood ( $5.03 \pm 0.41$ ), with a transient peak being observed on P7 ( $4.49 \pm 0.2$ ; Fig. 1).

In comparison to age-matched Nx rats, prenatal Hx lowered the E/NE ratio on E19 (-76%), P0 (-59%), P3 (-34%), and P7 (-36%; Fig. 1). This effect reflected primarily the higher proportion of NE in the Hx pups from E19 to P7, as well as the lower proportion of E.

In the case of the Nx control group, adrenal TH activity increased 43-fold from the time of birth until adulthood, with a transient decrease being observed between P0 and P3. The major elevations in this activity occurred between P3 and P7 (3.8-fold) and between P21 and adulthood (8.7-fold; Table 3).

In the Hx group, the TH activity increased progressively to reach a level of 40-fold at adulthood compared with the level at birth, but without the transient decrease observed on P3 in the Nx control pups. This increase occurred primarily between P7 and P14 (3.4-fold) and between P21 and adulthood (7.4-fold). In comparison to Nx control values, the TH activity was higher on P3 (+59%) and P14 (+122%) and lower on P68 (-26.6%) in the prenatal Hx animals (Table 3).

**Expression of TH and PNMT by the chromaffin cells.** In the normal rats, the TH and PNMT could first be detected

**Table 1.** Effect of prenatal hypoxia on the body weight and adrenal gland weight of rat pups

Group	Age of rats							
	E19	P0	P3	P7	P14	P21	P68	
Body weight (g)	Nx	2.769 $\pm$ 0.05 <i>n</i> = 10	5.838 $\pm$ 0.08 <i>n</i> = 10	8.688 $\pm$ 0.12 <i>n</i> = 15	15.229 $\pm$ 0.4 <i>n</i> = 14	30.474 $\pm$ 0.42 <i>n</i> = 15	45.376 $\pm$ 0.68 <i>n</i> = 15	363.273 $\pm$ 10.63 <i>n</i> = 11
	Hx	2.122 $\pm$ 0.08* <i>n</i> = 13	4.914 $\pm$ 0.27* <i>n</i> = 14	7.915 $\pm$ 0.33 <i>n</i> = 13	13.896 $\pm$ 0.36* <i>n</i> = 15	32.845 $\pm$ 0.75* <i>n</i> = 15	45.643 $\pm$ 0.82 <i>n</i> = 15	338.500 $\pm$ 7.86 <i>n</i> = 10
Adrenal weight (mg)	Nx	1.039 $\pm$ 0.06 <i>n</i> = 10	1.61 $\pm$ 0.12 <i>n</i> = 10	1.220 $\pm$ 0.08 <i>n</i> = 10	1.867 $\pm$ 0.08 <i>n</i> = 12	4.033 $\pm$ 0.29 <i>n</i> = 12	7.133 $\pm$ 0.36 <i>n</i> = 12	29.900 $\pm$ 1.42 <i>n</i> = 11
	Hx	0.889 $\pm$ 0.07 <i>n</i> = 10	1.010 $\pm$ 0.1* <i>n</i> = 10	1.055 $\pm$ 0.141 <i>n</i> = 10	2.020 $\pm$ 0.15 <i>n</i> = 10	5.764 $\pm$ 0.33* <i>n</i> = 14	7.564 $\pm$ 0.25 <i>n</i> = 11	26.040 $\pm$ 0.92* <i>n</i> = 10

Data are expressed as mean  $\pm$  SEM.

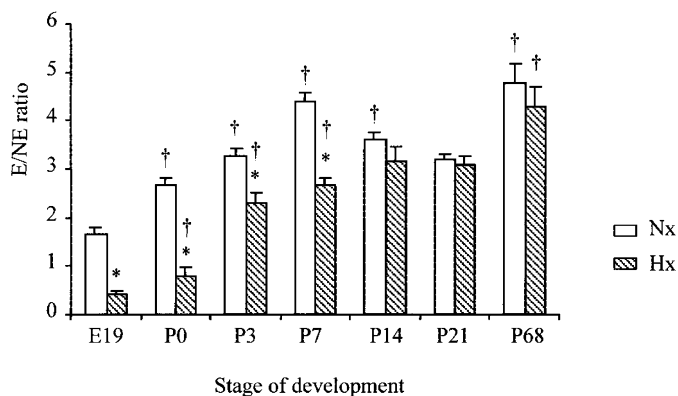
\* *p* < 0.05 represents significant difference between the Nx group and prenatal Hx group.

**Table 2.** Effect of prenatal hypoxia on the content of catecholamines (CA), NE, and E in the adrenal glands of rat pups

		Ages of rats						
Group		E19	P0	P3	P7	P14	P21	P68
CA (pmol/ gland)	Nx	462.4 ± 20.2 <i>n</i> = 11	1247.2 ± 64.4 <i>n</i> = 11	2585.9 ± 148.2 <i>n</i> = 10	5187.2 ± 256.2 <i>n</i> = 13	13707 ± 151.1 <i>n</i> = 12	21523 ± 534.7 <i>n</i> = 13	84462 ± 2926.5 <i>n</i> = 9
	Hx	321.5 ± 33.2* <i>n</i> = 14	1554.9 ± 66.6* <i>n</i> = 13	2618.4 ± 133.1 <i>n</i> = 9	5849.9 ± 398.7 <i>n</i> = 13	13647 ± 336.0 <i>n</i> = 9	21041 ± 1284 <i>n</i> = 10	81216 ± 5414 <i>n</i> = 6
NE (pmol/ gland)	Nx	168.4 ± 10.6 <i>n</i> = 11	313.9 ± 14.8 <i>n</i> = 11	594.4 ± 43.0 <i>n</i> = 10	952.4 ± 63.2 <i>n</i> = 13	2926.9 ± 118.3 <i>n</i> = 12	5077.8 ± 148.4 <i>n</i> = 13	14562 ± 1352 <i>n</i> = 9
	Hx	215.9 ± 20.3* <i>n</i> = 14	888.2 ± 44.9* <i>n</i> = 13	786.2 ± 54.7* <i>n</i> = 9	1594.3 ± 75.1* <i>n</i> = 13	3231.9 ± 261.3 <i>n</i> = 9	5122.8 ± 331.6 <i>n</i> = 10	15268 ± 1962 <i>n</i> = 6
E (pmol/ gland)	Nx	280.1 ± 12.5 <i>n</i> = 11	833.3 ± 26.7 <i>n</i> = 11	1950.8 ± 108.1 <i>n</i> = 10	4174.7 ± 187.2 <i>n</i> = 13	10512.1 ± 84.4 <i>n</i> = 12	16256 ± 471.1 <i>n</i> = 13	69405 ± 2205 <i>n</i> = 9
	Hx	91.7 ± 15.1* <i>n</i> = 14	718.4 ± 44.2* <i>n</i> = 13	1804.9 ± 116.1 <i>n</i> = 9	4272.9 ± 302.4 <i>n</i> = 13	10048.4 ± 167.2* <i>n</i> = 9	15745 ± 1045.8 <i>n</i> = 10	65451 ± 3794 <i>n</i> = 6

Data are expressed as mean ± SEM.

\**p* < 0.05 represents significant difference between the Nx group and prenatal Hx group.



**Figure 1.** Effect of long-term prenatal exposure to Hx on the ontogenesis of the E/NE ratio in the adrenal glands of rat on E19, P0, P7, P21, and P68. The ratios were determined from the contents expressed as picomoles per gland (mean ± SEM). \**p* < 0.05 denotes a significant difference between the Nx (white bars) and prenatal Hx groups (cross-hatched bars). †*p* < 0.05 denotes a significant difference between two consecutive stages of development.

histochemically on E19. The chromaffin cells were found to be clustered, as in the adult adrenal medulla, but these clusters were somewhat more scattered and the neuronal and interstitial tissues more abundant. The relative surface areas occupied by these cells increased during development, and the chromaffin cells became progressively more clustered, as in the adult adrenal. The medullary portion of this organ became a distinct entity between P7 and P21 of development (Fig. 2). On E19 and at birth, the relative area occupied by TH-ir<sup>+</sup> (28.8 ± 2.4% and 39.4 ± 3.2%, respectively) or by PNMT-ir<sup>+</sup> (33.9 ± 4.6% and 27.7 ± 2.7%, respectively) were lower than on P21. On P7 and P21, TH-ir<sup>+</sup> chromaffin cells constituted the largest portion of the medulla (94.5 ± 2.2% and 71.7 ± 4.1%, respectively), whereas PNMT-ir<sup>+</sup> chromaffin cells occupied a smaller surface (58.4 ± 0.8% and 41.7 ± 7.8%, respectively; Fig. 3).

Prenatal Hx altered the ontogenesis of the expression of the TH and PNMT proteins in the rat adrenal medulla (Fig. 3). Indeed, in the prenatal Hx group, the relative surface occupied by TH-ir<sup>+</sup> chromaffin cells was decreased at the late embryonic stage (E19) and at birth (−50% and −27%, respectively) compared with the Nx controls. On E19, there was also a significant decrease (−46%) in the relative surface area cov-

ered by PNMT-ir<sup>+</sup> chromaffin cells. These decreases were still present on P7, both at the protein level (−27% in the case of the TH protein and −34% for PNMT) and at the mRNA level of gene expression (−20% for TH mRNA and −51% for PNMT). Indeed, on P7 decreases in the ODs of both TH mRNA (0.612 ± 0.014 in the case of Nx pups versus 0.488 ± 0.012 for Hx pups) and PNMT mRNA (0.480 ± 0.013 for Nx pups versus 0.233 ± 0.011 for Hx pups) were observed. By P21, the expression of TH and PNMT in the adrenal of prenatal Hx pups was the same as in the Nx control group (Fig. 3).

## DISCUSSION

In the current study, the main finding was that long-term prenatal Hx, lasting for 2 wk preterm, results in alterations in the maturation of the adrenal medulla that persist from the end of gestation until the first 2 wk of postnatal life and even into adulthood. These alterations in maturation involved different levels of catecholamine biosynthesis, *i.e.* adrenal catecholamine content, TH activity, and the levels of TH and PNMT proteins and mRNAs.

The exposure to prenatal Hx used here is an appropriate model for the Hx associated with numerous pathologic situations. Our protocol involves exposure to moderate Hx (equivalent to living at an altitude of approximately 5500 m) during three fourths of the gestational period. The weight gain of the pregnant rats and the average litter size were both reduced by such prenatal Hx, perhaps because of a reduction in food intake (31). In the present study, we observed an increase in the placental weight and in the placental to fetal body weight ratio in the Hx group, a specific indicator of maternal Hx rather than low maternal protein intake (18). Therefore, the pronounced weight reductions of offspring on E19, P0, P3, and P7 did not reflect restricted maternal food intake, but are rather related to the maternal exposure to Hx. To avoid possible effects of Hx on the quality of the milk, Hx pups were suckled by Nx dams.

In the Hx fetuses, the adrenal content of total catecholamines was decreased, as was the E/NE ratio and the expression of TH and PNMT, indicating that the synthesis of catecholamines, and especially of E, is reduced after prenatal Hx. These pups should thus be more vulnerable to the stress associated with

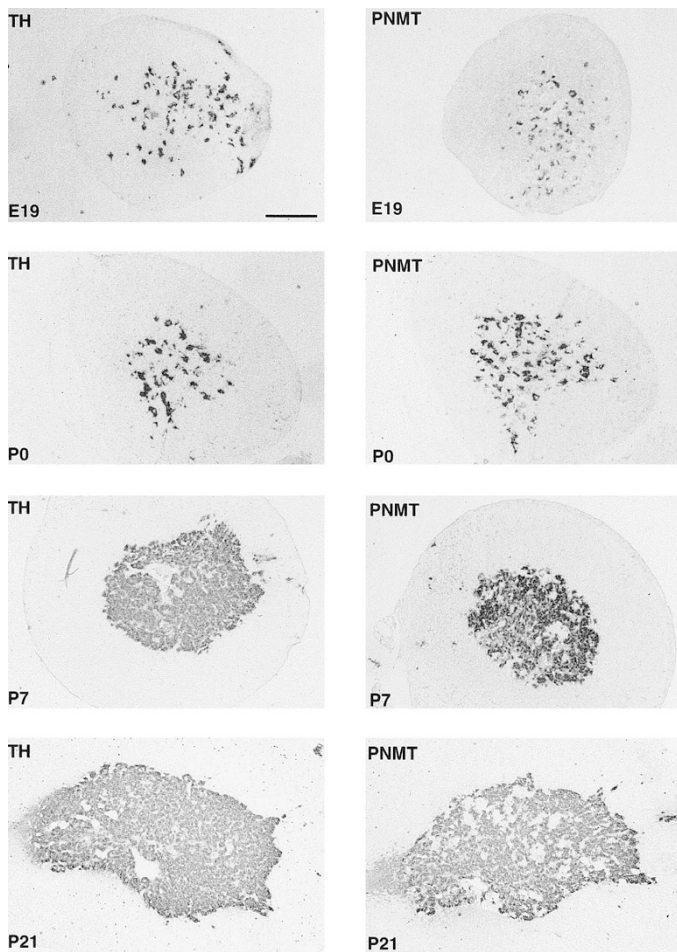
**Table 3.** Effect of prenatal hypoxia on the accumulation of DOPA in the adrenal glands of rat pups treated with NSD-1015

		Age of rats					
Group		P0	P3	P7	P14	P21	P68
DOPA (pmol/20 min/gland)	Nx	6.41 ± 1.01 <i>n</i> = 10	3.91 ± 0.46† <i>n</i> = 10	15.32 ± 1.30† <i>n</i> = 12	23.49 ± 2.73† <i>n</i> = 12	31.73 ± 4.14† <i>n</i> = 8	274.55 ± 26.14† <i>n</i> = 9
	Hx	4.93 ± 0.78 <i>n</i> = 10	6.24 ± 1.01* <i>n</i> = 10	15.31 ± 1.48† <i>n</i> = 10	52.21 ± 7.38*† <i>n</i> = 9	27.16 ± 2.14† <i>n</i> = 10	201.52 ± 15.17*† <i>n</i> = 10

Data are expressed as mean ± SEM.

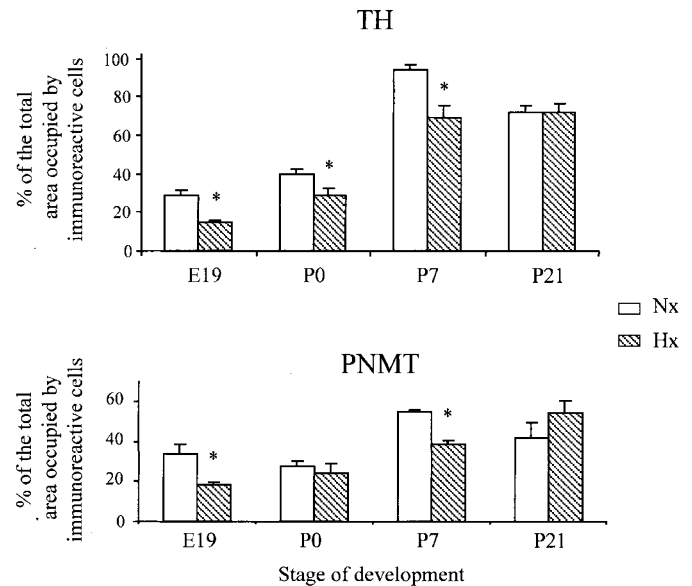
\* *p* < 0.05 represents significant difference between the Nx group and prenatal Hx group.

† *p* < 0.05 represents significant difference between two successive ages.



**Figure 2.** Immunohistochemical staining of TH and PNMT in the adrenal gland of normal rats on E19, P0, P7, and P21. Consecutive adjacent paraffin sections (4 μm thick) of the adrenal gland were immunolabelled (avidin biotin–peroxidase complex) for TH (1/1500) and PNMT (1/1000) as described in the “Methods.” Scale bar = 200 μm.

birth. At the time of birth, their higher level of catecholamines and unchanged TH activity suggest that the release of catecholamines might be defective in prenatal Hx pups. Because the catecholamine surge at birth is essential for the maintenance of cardiovascular and metabolic homeostasis (1, 4), it appears likely that these prenatal Hx pups are less capable of adapting to the neonatal situation. On P3, TH activity was higher in the Hx pups, reflecting the fact that these pups did not demonstrate the decrease in this activity observed in Nx pups after birth. Such a decrease between P0 and P2 in control pups has also been observed in the case of the TH mRNA (7). Thus,



**Figure 3.** Effect of long-term prenatal exposure to Hx on the ontogenesis of the expression of TH and PNMT proteins in the adrenal medulla of rats on E19, P0, P7, and P21. The data are expressed as percentage of the area occupied by positively immunoreactive cells in comparison with the total area of the adrenal medulla (mean ± SEM). The number of animals examined at each developmental age was six. \**p* < 0.05 denotes a significant difference between the Nx (white bars) and prenatal Hx (cross-hatched bars) groups.

the adrenals of Hx pups remained at a high level of activity during the first days of postnatal life.

With regards to cells that are positively immunoreactive for the TH and PNMT proteins, prenatal Hx altered the histologic maturation of the adrenal medulla. In the Hx group, the TH-ir<sup>+</sup> and PNMT-ir<sup>+</sup> cells were more scattered than in the control group at the same stage of development. Prenatal Hx induced a reduction in the proportion of TH and PNMT immunoreactive cells in the adrenal medulla on E19, P0, and P7, together with a decrease in the level of the TH and PNMT mRNAs on P7 and a decrease in the E content, indications of immaturity.

This impaired expression of the TH and PNMT enzymes emphasizes the fact that the fetal and neonatal adrenals of the prenatal Hx pups are compromised in their ability to synthesize or to secrete E and NE. Thus, prenatal exposure to Hx causes a delay in the maturation of the medullary portion of adrenal gland, both in terms of the level of related proteins and their mRNAs and catecholamine production. Our findings demonstrate that decreased levels of TH protein and mRNA were associated with increased TH activity, whereas the decreased levels of PNMT protein and mRNA were associated with a

similar reduction in the proportion of E during the first 2 wk of postnatal life. Thus, the levels of TH and PNMT proteins appear to be differentially regulated.

This retarded maturation with respect to TH and PNMT might result from an altered involvement of glucocorticoids. Indeed, it is well known that PNMT and TH are under the control of glucocorticoids (32, 33) released from the maturing adrenal cortex or maturation of the glucocorticoid receptors in the medulla (7). PNMT is also controlled, to a lesser extent, by impulses from the splanchnic nerve to the adrenal medulla. Glucocorticoids play an essential role in triggering the differentiation of noradrenergic sympathoadrenal precursor cells to the adrenergic chromaffin cells, after a functional system of glucocorticoid receptors has been established (34, 35). In the adult rats, high glucocorticoid levels induce the expression of PNMT and TH mRNA (36–38). In contrast, in the case of fetal adrenals, it has been suggested that glucocorticoids exert an inhibitory effect on the expression of both PNMT mRNA and protein as well as on E synthesis (39), thus increasing the store of NE (40). Retardation of the maturation of expression of PNMT and TH as a consequence of prenatal Hx might thus reflect an alteration in the control of catecholamine biosynthesis by glucocorticoids. Long-term exposure to Hx increased plasma levels of glucocorticoids (41). Normally, the fetus is protected from maternal glucocorticoids by placental inactivation with 11 $\beta$ -OHSD. Moreover, maternal glucocorticoid excess induced a partial deficiency of the placental barrier to glucocorticoids (42, 43). There was a positive correlation between placental 11 $\beta$ -OHSD activity and fetal weight, and an inverse correlation between placental 11 $\beta$ -OHSD and placental weight (42). Our data—low birth weight, large placenta—might suggest that prenatal Hx reduced the efficiency of the placental glucocorticoid barrier, thus increasing fetal glucocorticoid exposure. The alterations observed in the adrenals of rats subjected to prenatal Hx might be related to increased fetal exposure to glucocorticoids.

Moreover, in Hx pups on P7, the peak E/NE ratio and the overexpression of TH and PNMT exhibited by the control animals were lacking. However, at a later time point, *i.e.* on P14, a transient TH hyperactivity was detected in the Hx pups. The increases in the E/NE ratio and expression of related proteins found on P7 in the control animals occur concomitantly with the functional splanchnic innervation of the adrenal (44). Indeed, the trophic input from splanchnic nerve leads to increased catecholamine biosynthesis and storage (45). Therefore, the disturbed pattern of adrenal maturation observed in the Hx pups might result from an alteration in timing of the onset of splanchnic innervation.

In adult rats, the decreased TH activity, together with the unchanged catecholamine content after prenatal Hx, indicate reduced catecholamine release and impaired adrenal function on the whole. These data indicate that prenatal Hx disturbs the ontogenesis of adrenal function by altering processes involved in the synthesis or release of NE and E. The major effects in this respect were exhibited during the perinatal period, but this impairment persisted into adulthood. Similarly, altered adrenomedullary development has been previously reported in connection with intrauterine growth retardation (46), prenatal

exposure to dexamethasone (47), and placental restriction (48). All these previous findings together with our results indicate that insult during gestation impaired adrenal function.

The present findings are of significance with respect to neonatal pathology in light of the crucial involvement of catecholamines in autoresuscitation (49). Such disturbances in adrenal function could impair adaptation to a new neonatal environment. Alteration of adrenal function in newborns may also contribute to the pathogenesis of perinatal morbidities. Deficient mobilization of the catecholamine stores in the adrenals of sudden infant death syndrome victims has been reported (50). At adulthood, the effect of prenatal Hx still persists and may reduce the ability to adapt to various environmental stresses.

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