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Expression of *c-fos*, Tyrosine Hydroxylase, and Neuropeptide mRNA in the Rat Brain around Birth: Effects of Hypoxia and Hypothermia

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ABSTRACT

Arousal at birth is likely to be accompanied by changes in gene expression patterns in the brain. We analyzed the expression levels of genes that may be involved in neonatal adaptation. We have also tried to dissect the effect of hypoxia and hypothermia, two components that may play a role in gene expression at birth. Therefore, we analyzed the expression patterns of the *c-fos*, tyrosine hydroxylase, enkephalin, preprotachykinin-A, and neuropeptide Y genes in various brain regions of rat pups at various time points after cesarean section under normal conditions and after exposure to hypoxia and hypothermia. We found that *c-fos* RNA was up-regulated transiently after birth in neocortex, midbrain, and pons-medulla with a maximum of 30 min after cesarean section, and that this transient increase was not further augmented by hypoxia and hypothermia. The expression patterns of the other genes were not significantly altered, with the exception of a very slight increase in tyrosine hydroxylase RNA levels. We discuss tentative mechanisms for the transient increase in *c-fos* expression and the possible involvement of catecholamines in this process. (***Pediatr Res* 37: 15–20, 1995**)

Abbreviations

TH, tyrosine hydroxylase
P1, P2 etc., number of days after birth
PPT-A, preprotachykinin-A
NPY, neuropeptide Y
d.p.c., days postcoitum

Birth represents a major transition in many important body functions, *e.g.* digestion, metabolism, respiration, and temperature regulation (for review see Ref. 1). The newborn infant is aroused and awakened from its mainly asleep state in the uterus, a process probably related to the activation of the locus coeruleus (2, 3), and accompanied by a surge of catecholamines and other neuroendocrine hormones (4).

Several changes in gene expression patterns around birth have been recorded. PPT-A RNA was found to be up-regulated about 4-fold in the respiratory nucleus tractus solitarius after birth in rabbit pups (5, 6). This is of interest because substance P, which is translated from one of the mRNA from the PPT-A gene, is the dominant neuropeptide in these main respiratory nuclei and acts as a respiratory stimulatory agent (7). This change in expression was not observed in pups who were not breathing, and the expression of PPT-A RNA was not changed in nonrespiratory brain nuclei around birth (6). In another study the RNA for TH, which is the rate-limiting enzyme in the catecholamine synthesis, was shown to increase about 4-fold in the adrenal medulla after 12 h in newborn rats (8). Similar increases were observed for RNA from the dopamine β-hydroxylase and NPY genes in the adrenal medulla (8).

Expression of the protooncogene *c-fos*, one of the immediate early genes, is known to be increased at birth in several tissues, including brain (9). This increased expression is attributed to the external stimuli after birth and possibly also to the catecholamine surge at birth (9). Levels of *c-fos* RNA were also found to be increased in corticotropin-releasing hormone neurons in the fetal sheep hypothalamus just before birth, suggesting that *c-fos* may be involved in the onset of labor (10). Expression of *c-fos* has also been shown to occur concomitant with brain seizures, which may argue for a role of *c-fos* in neuronal activity (11).

In this study we wanted to investigate in more detail the expression of *c-fos*, TH, PPT-A, enkephalin, and NPY genes in newborn rats. Expression levels were analyzed separately from three different brain regions: neocortex, striatum-diencephalon, and pons-medulla. These regions were selected because, first, the neocortex is postulated to be

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aroused at birth, second, hypothalamus is part of the striatum-diencephalon samples and, finally, autonomic functions are largely regulated in the pons-medulla. We also dissected the effects of hypoxia and hypothermia, which often occur in relation to birth.

METHODS

Animals. Sprague-Dawley rats were used throughout the experiments, and the animals were cared for in compliance with the National Institutes of Health guidelines for animal experiments. Pregnant rats were killed on the 22nd d after fertilization (21.5 d.p.c.) and the pups delivered in less than 5 min by cesarean section. To establish the expression profiles transiently after birth, some pups were killed immediately and others after 10 min, 30 min, 1 h, 2 h, and 4 h under normoxic conditions at 38°C. All pups were constantly observed and were breathing well after 30 min. To study the influence of hypoxia and hypothermia, a second set of experiments was performed and pups, which were delivered by cesarean section at 21.5 d.p.c., were divided into three groups and subjected to three experimental settings. One group was transferred within 1 min after delivery to 38°C under normoxic conditions as control, the second group was transferred to 38°C in a hypoxic environment (9% oxygen in nitrogen), and the third group was transferred to 22°C in a normoxic environment. After 1 h the pups were immediately killed and RNA extracted (see below). Less than 5% of the pups died during hypoxia and hypothermia and these animals were not included in the study. For each time point under the different conditions, three independent experiments, each with eight to 16 animals, were performed.

Preparation of tissue and RNA extraction. The brains of the killed animals were dissected out and quickly divided into three areas: neocortex, striatum-diencephalon, and pons-medulla. In each experiment, the samples from each area were frozen on dry ice and pooled (n = 8–16). The tissue samples were homogenized with a Polytron in 4 M guanidine isothiocyanate, 0.1 M β-mercapthoethanol, 0.025 M sodium citrate, pH 5.5, layered on top of a CsCl/citrate cushion and centrifuged at 15°C in a Beckman SW41 rotor at 35,000 rpm for 21 h (12). The total RNA was dissolved and the polyA$^+$ fraction purified by conventional oligo-dT column chromatography (13). The amounts of ribosomal RNA still present in the polyA$^+$ fraction after this purfication procedure were very reproducible and were used to assess the integrity of the RNA and to quantitate loadings on the Northern blot gels (see below). The concentration of RNA was estimated by spectrophotometry at 260 nm. RNA from each sample (10 μg) was electrophoresed in 1% agarose gels containing 0.7% formaldehyde and 500 ng/mL ethidium bromide and photographed under UV-light. The intensity in the ethidium bromide staining in the 28S RNA band was quantitated using a Leica Quantimet 570 system (Cambridge Instruments, Cambridge, UK) and used to normalize the amounts of RNA loaded (see below).

Northern blot hybridization. After gel electrophoresis, the RNA was transferred to nitrocellulose filters as previously described (12). After baking in vacuum for 2 h at 80°C the filters were prehybridized for 1 h at 43°C in 4 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 40% formamide, 1 × Denhardt's solution (50 × Denhardt's solution = 5 g/L of BSA, polyvinylpyrrolidone, and Ficoll), 10% dextran sulfate, and 150 μg/mL of denatured sheared salmon sperm DNA for 1 h at 43°C to block nonspecific binding of the probe. The prehybridization solution was then replaced with the hybridization solution (same composition as the prehybridization solution) containing the probe, labeled by nick translation (14) to a sp act of 5×10^8 dpm/μg at a concentration of 2×10^6 dpm probe/mL hybridization solution. Rat cDNA probes for the TH, PPT-A, and NPY genes, a mouse cDNA clone for *c-fos*, and a human clone for the enkephalin gene were used as probes. After hybridization for more than 16 h at 43°C the filters were washed at high stringency (final wash = 0.1 × SSC, 0.1% SDS, 2 × 30 min at 55°C) for the TH, PPT-A, and NPY probes and at moderate stringency (final wash = 0.2 × SSC, 0.1% SDS, 2 × 30 min at 50°C) for the *c-fos* and enkephalin probes. The filters were then exposed to Kodak X-AR5 film at −70°C for various time periods to ensure that the signal on the x-ray film was in the linear range. The autoradiograms were quantified using a Shimadzu CS-9000 densitometer (Shimadzu, Kyoto, Japan) or a Leica Quantimet 570 image analysis system. To compensate for variations in the loading of RNA, the hybridization signal was normalized to the amounts of 28S RNA estimated from the ethidum bromide staining of the gel. To compare data from the individual experiments in the presentation of the data, the hybridization signal for 0 h after birth was set to 1 and the results from other time points in the same experiment were recalculated accordingly. In some cases, the RNA yield from an experiment was enough to run more than one gel, and the results from the resulting autoradiograms were averaged after they had been normalized separately. As an additional control for standardization, all filters were stripped and rehybridized with a rat β-actin probe at high stringency. The results from this rehybridization were generally in very good agreement with the 28S RNA data (data not shown).

Statistics. The nonparametric Mann-Whitney test was used to statistically compare the different data. $p < 0.05$ was regarded as significantly different.

RESULTS

We used Northern blot hybridization to analyze the levels of the different RNA in rat pups during the first hours after cesarean section and after exposure to hypoxic and hypothermic conditions. In all cases, the probes identified mRNA of the expected lengths: 2.2 kb (*c-fos*), 1.9 kb (TH), 1.5 kb (enkephalin), 1.0 kb (NPY), and 1.3 kb PPT-A (Fig. 1). As a control, a filter was rehybridized with an actin probe, which identified the expected 2.0 kb mRNA.

Gene expression after cesarean section. Rat pups were delivered by cesarean section at 21.5 d.p.c., and expression of *c-fos*, TH, enkephalin, NPY, and PPT-A was analyzed immediately (0 h) and after 10 min, 30 min, 1 h, 2 h, and 4 h in a normoxic, thermoneutral (38°C) environment. The most notable difference in gene expression was a transient increase in

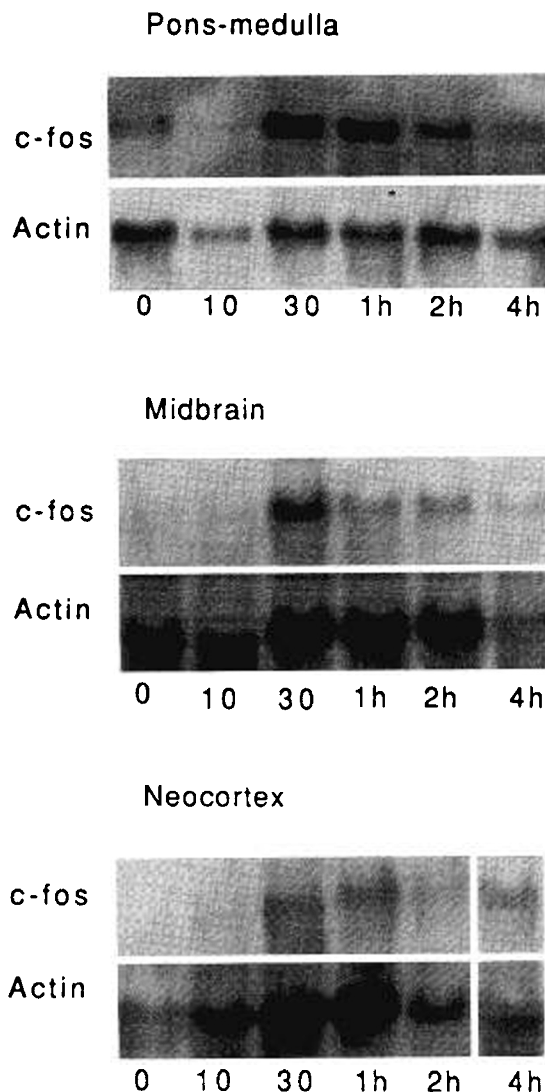

Figure 1. Northern blot analysis of brain RNA hybridized with the different probes. Ten μg of polyA$^+$ RNA from neocortex 1 h after birth were hybridized with probes for actin, *c-fos*, enkephalin, NPY, PPT-A, and TH. To the left, the ethidium bromide staining of a gel is shown, and the positions of the 28S and 18S rRNA bands are indicated.

c-fos RNA expression in both neocortex, striatum-diencephalon, and pons-medulla (Fig. 2). The highest levels in all three brain regions were recorded at 30 min with median values 2.5 to 4.5 times as high as in animals killed immediately after cesarean section. This increase was statistically significant in all three brain areas as determined by the Mann-Whitney test ($p < 0.05$). After 1 h, the *c-fos* RNA levels had decreased somewhat, but the median *c-fos* RNA levels in neocortex and pons-medulla were still higher than at 0 h. At 4 h, the *c-fos* RNA levels had returned to the same level as at 0 h in all three brain regions (Fig. 2).

Changes in expression of the other genes were not as pronounced as for *c-fos*, but a slight increase in TH expression was observed, although it was not statistically significant (data not shown). Similarly, the expression of NPY and enkephalin RNA increased slightly in some of the experiments (data not shown). RNA levels for PPT did not undergo detectable changes in this study (data not shown).

Gene expression under hypoxic and hypothermic conditions. To dissect the effects of the transition at birth on gene expression in more detail, rat pups delivered by cesarean section were exposed to hypoxic or hypothermic conditions. When analyzed 1 h after exposure to hypoxia (9% oxygen in nitrogen at 38°C), the median *c-fos* levels showed an increase in neocortex and pons-medulla compared with the levels at 0 h ($p < 0.05$), although the increase was less dramatic in striatum-diencephalon (Fig. 3). The increase was comparable to that seen 1 h after cesarean section under normoxic conditions. A single experiment after 30 min in hypoxia showed even higher *c-fos* levels in striatum-diencephalon and pons-medulla (data not shown), which were comparable to those seen under normoxic conditions. The TH RNA levels were somewhat increased after hypoxia in neocortex and striatum-diencephalon, although the increase was not statistically significant.

The data from exposure to hypothermia (22°C in a normoxic environment) were very similar to the hypoxic situation (Fig. 3). A statistically significant increase in *c-fos* RNA levels, compared with the 0 h control ($p < 0.05$), was seen after 1 h of exposure to hypothermia in neocortex and striatum-diencephalon. The increase was comparable to that seen after 1 h in normal temperature. In a single experiment, a similar increase was seen after 30 min.

Our experiments did not show any significant changes in the RNA levels for the other genes after exposure to hypoxia and hypothermia (data not shown). Although the median TH RNA levels were three times higher in the hypoxic compared with the normoxic animals after 1 h, the difference was not significant (data not shown).

DISCUSSION

The aim of this study was to analyze changes in the expression of some neuroactive genes possibly involved in the arousal and onset of homeostatic functions at birth. It is generally assumed that adaptations to the new environment largely occur in the CNS, and therefore we analyzed three anatomically and functionally distinct areas: neocortex, diencephalon-striatum, and pons-medulla. We also wanted to examine the transition to the extrauterine environment in more detail and therefore analyzed the effects of 1 h of exposure to hypoxia, hypothermia, and normoxic, isothermic conditions after birth.

The most important finding was that *c-fos* RNA levels are transiently increased after birth, both after cesarean section under normal conditions and after cesarean section followed by hypoxia and hypothermia. This transient increase may be important for gene regulation at birth because the *c-fos* protein is a transcription factor that is involved in activating other genes, *e.g.* collagen, collagenase, and *c-myc*, by binding to control elements in their promotors (see Ref. 15 for review). Previous studies in the mouse have demonstrated an upregulation of *c-fos* RNA after birth in several tissues, including brain (9, 16). In the study by Kasik *et al.* (9), *c-fos* RNA was detected in the brain on the day of birth and P1 and then increased in P3 and P5 animals. Our study extends this observation by showing that there is also a transient peak of *c-fos*

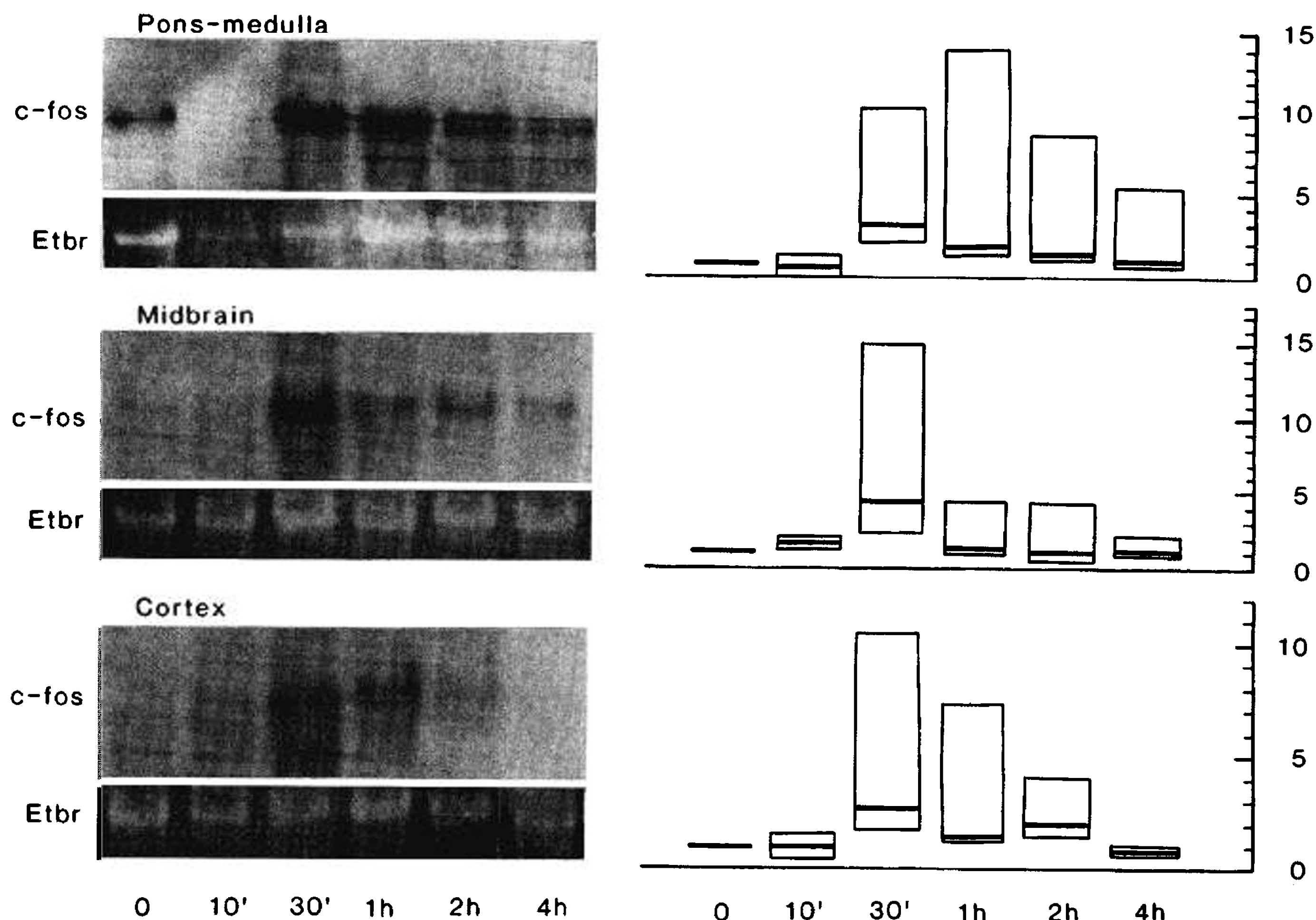

Figure 2. Northern blot analysis of brain RNA hybridized with the *c-fos* probe at different time points after cesarean section. To the left is shown a Northern blot analysis of 10 μg of polyA$^+$ RNA from neocortex, striatum-diencephalon, and pons-medulla at 0, 10, 30, and 60 min and 2 and 4 h after cesarean section hybridized with the *c-fos* probe. The ethidium bromide staining (*EtBr*) of the 28 S rRNA band from the same lanes is shown below each blot. To the right, the densitometric scannings of three independent experiments are shown (one of which is shown to the *left*). The maximum, minimum, and median (*thick horizontal bar*) values are plotted. The values at 0 min were set to 1 and all other values recalculated accordingly (see Methods).

expression 30 min after cesarean section. This transient peak is similar to the *c-fos* expression profile in mouse liver after cesarean section, which showed the highest levels of *c-fos* RNA 60 min after cesarean section (16). Although we cannot strictly rule out a species difference in *c-fos* expression between rat and mouse, it seems likely that *c-fos* in brain has a biphasic mode of expression with a transient peak within 1 h after birth followed by the previously described slower surge over the first few postnatal days. This transitory increase of *c-fos* might be sufficient to trigger mechanisms involved in the neonatal adaptation.

Our experiments showed that hypoxia and hypothermia did not further elevate the transient increase in *c-fos* expression, because the levels of *c-fos* RNA were approximately the same in the different experimental settings. It has previously been shown that the *c-fos* gene can be activated by a specific transcription factor, the serum response factor, which binds to the serum response element in the *c-fos* enhancer (see Ref. 15 for review). The situation in neonatal induction may, however, be different and involve also binding of another transcription factor (16). It will be interesting to establish which combination of transcription factors mediates the transient induction after birth.

Our data suggest that the physiologic stimulus of *c-fos* expression is derived from the transition from intra- to extrauterine environment *per se* rather than from changes in oxygen and temperature conditions. The use of cesarean section also eliminated the possible effects on *c-fos* induction caused by mechanical stress on the fetus during vaginal delivery. The *c-fos* induction could be caused by a general increase of the somatosensory input and subsequently augmented neuronal activity in the brain. In this context, it is interesting to recall that synaptic activity can act as an inducer of *c-fos* expression in hippocampus (11).

Catecholamines have also been considered as a possible inducer of *c-fos* expression, because their levels are markedly elevated at birth (4, 17). However, it is likely that the situation is more complex, because the surge of catecholamines may partly result from the oxygen deficiency associated with birth (4), and the *c-fos* RNA levels were not further increased at hypoxic conditions. Our findings of only a relatively small increase in TH RNA levels may suggest that the increased

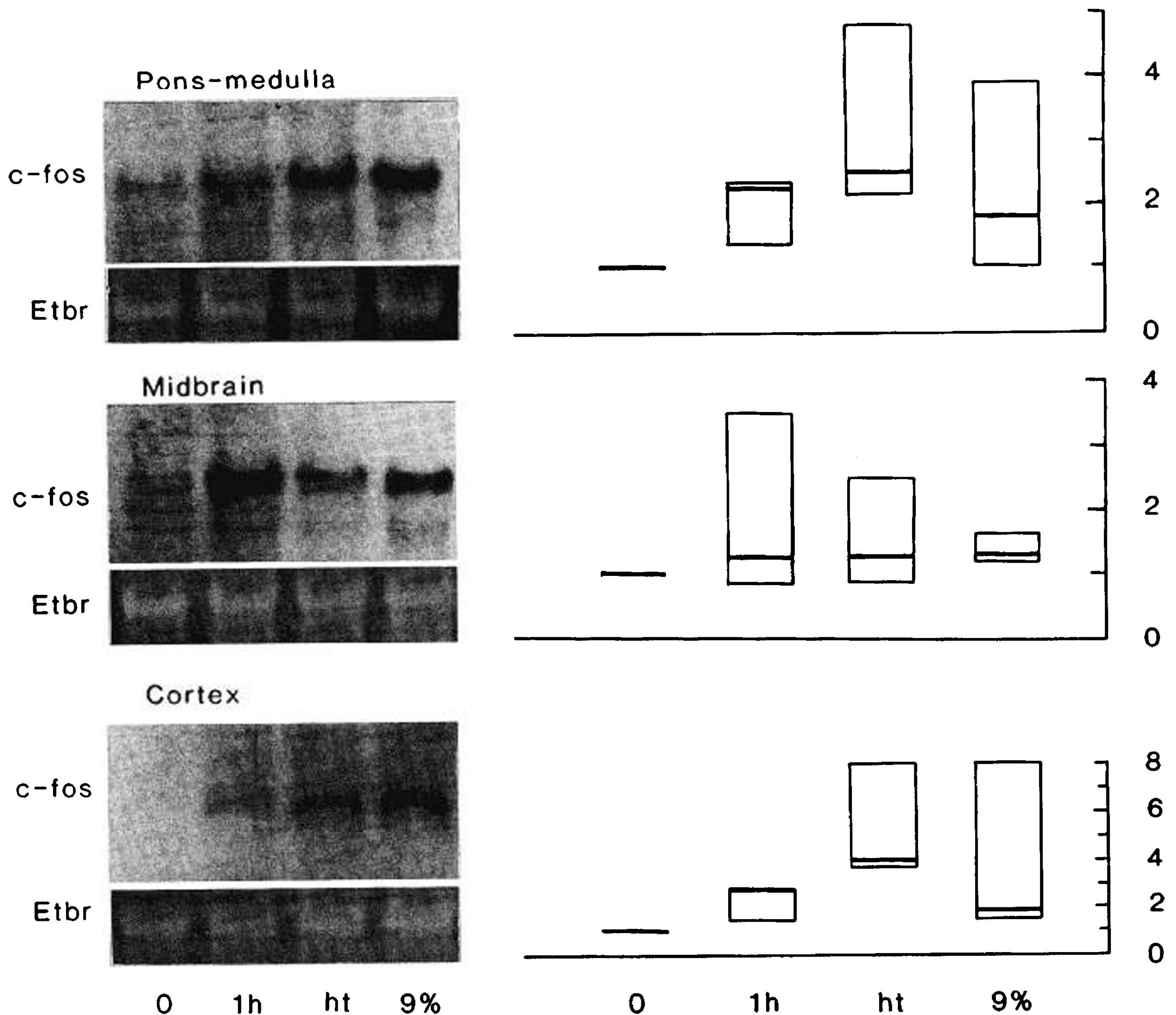

Figure 3. Northern blot analysis of brain RNA after hypoxia and hypothermia hybridized with the *c-fos* probe. To the left is shown a Northern blot analysis of 10 μg of polyA^+ RNA from neocortex, striatum-diencephalon, and pons-medulla at 0 min and after 1 h at 38°C under normoxic conditions (*1h*), after 1 h in a normoxic environment at 22°C (*ht*), and after 1 h at 9% oxygen in nitrogen at 38°C (*9%*). The ethidium bromide staining (*EtBr*) of the 28 S rRNA band from the same lanes is shown below each blot. To the right, the densitometric scannings of three independent experiments are shown (one of which is shown to the left). The maximum, minimum, and median values are plotted. The values at 0 min were set to 1 and all other values recalculated accordingly (see Methods).

turnover of catecholamines in the newborn brain (3) is not coupled with the transcriptional control of the TH gene, but may involve posttranscriptional regulation of TH protein levels. We also cannot exclude that a surge in TH occurs later during development. It should also be emphasized that pups in all experiments were delivered by cesarean section and that the absence of labor leads to a smaller surge in catecholamines.

We observed very small, statistically insignificant changes in expression of the various neuropeptide RNA, including PPT-A RNA. The latter finding was somewhat surprising given the data from newborn rabbit pups, in which PPT-A RNA has been shown to increase about 4-fold at the day of birth (6). This difference may be explained by a species difference between rat and rabbit pups. Rabbit pups, in contrast to rat pups, are precocial developers, and more closely resemble human infants with regard to the maturation of sleep-wakefulness cycles (18). The latter possibility could be addressed by analyzing PPT-A levels in rats at different time points after birth.

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