Hypoxia-induced Long-term Increase of Dopamine and Tyrosine Hydroxylase mRNA Levels

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Dedicated to professor MUDr. Stanislav Trojan, DrSc. to the 70th birthday

Abstract: The aim of the present study was to determine hypoxia-induced changes in the long-term expression of tyrosine hydroxylase (TH) mRNA and the steady-state dopamine (DA) levels in rat mesencephalic cell cultures. The cultures were exposed to hypoxia during the early developmental period, and DA content and TH mRNA expression were determined on day *in vitro* (DIV) 14. Hypoxic exposure of 5-day-old cultures resulted in increased DA (control 89.9 ± 8.9, hypoxia 135.8 ± 23.7 pg/μg protein) and TH mRNA (control 37.3 ± 4.7, hypoxia 143.1 ± 49.4 pg/μg RNA) levels. To analyze the involvement of hypoxia-inducible factor-1 (HIF-1) in these changes, we studied its activation using reporter gene. Hypoxia caused a 3-fold increase in HIF-1 activity. Our data suggest that hypoxia/ischemia during the putative critical developmental period of neurons may determine the tyrosine hydroxylase gene expression and, consequently, the development of the dopaminergic system.

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Introduction

Perinatal hypoxia is known to cause a significant deficit in subsequent brain development manifested as long lasting neurological, cognitive and behavioral dysfunction [1, 2]. Such dysfunction is largely associated with changes in the properties of dopaminergic neurons [3, 4]. In previous studies, we investigated long-term posthypoxic changes in dopaminergic cells using the mesencephalic cell culture model [5, 6]. Mesencephalic and diencephalic cells prepared from rat fetuses on gestational day 14 and exposed to hypoxia during *in vitro* days (DIVs) 1–3 responded with long-term increases in both dopamine (DA) content and DA release. In principle, two mechanisms may be responsible for this long-term increase in DA content: (a) an increase in the number of tyrosine hydroxylase immunoreactive (TH-IR) neurons, and (b) a long-term enhancement of the transcription of TH as the main rate-limiting enzyme in DA synthesis [7]. Using the same model, we found that the number of TH-IR cells remained unchanged [5]. These results suggest that a single hypoxic exposure of dopaminergic cells during the putative critical developmental period led to a permanent increase in the steady-state mRNA level.

To test this hypothesis we studied the effect of a single hypoxic exposure at two different time periods during the early developmental period (DIVs 2 and 5) on the DA and TH mRNA levels in mature (DIV 14) mesencephalic neurons.

It is known that hypoxia up regulates the TH mRNA level by binding several transcription factors to the promoter region of the TH gene [8]. An important transcription factor activated by oxygen deficiency is hypoxia inducible factor-1 (HIF-1). HIF-1 is a heterodimer protein consisting of HIF-1a and HIF-1b subunits. Both subunits belong to the basic helix-loop-helix family of transcription factors [9] and have an important role to play during embryonic development [10]. It could be assumed that differential activation of HIF-1 by hypoxia is involved in the long-term increase in TH mRNA levels. To elucidate this question we determined the activation of HIF-1 after hypoxia on DIVs 2 and 5.

Materials and methods

Cell culture and exposure to hypoxia. Mesencephalic cultures were prepared from 14-day-old Wistar rat embryos as described previously [11]. Cell suspension was applied to poly-L-lysine-coated 48-well plates (seeding density 250,000 cells/cm^2 in 250 μl) and maintained up to DIV 14. Cultures were exposed to 24-h hypoxia on DIVs 2 and 5 in a Billup's chamber as described elsewhere [12]. In brief, the chamber was perfused with a calibrated gas mixture of 5% CO_2 and 95% N_2 (AGA Gas GmbH, Bottop, Germany). After a 15-min perfusion at a flow rate of 20 l/min the atmosphere contained 5–10 mm Hg oxygen. In the medium, a steady-state level of 10–20 mm Hg oxygen was reached after 30-min incubation and was maintained for up to 24 hours.

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Hypoxia-inducible factor-1 (HIF-1). To study HIF-1 activity we used a luciferase reporter construct based on the pGL3-Promoter Vector (Promega). The HIF-1 responsive vector, termed pHBE (HBE = HIF-1 Binding Element), was constructed by cloning three HIF-1 specific binding sites from the EPO gene in the Bgl II site of the enhancer region of the SV 40 promoter of the pGL3 [13]. A wild-type pGL3 vector was used as the control for transfection efficiency. Transfection of primary mesencephalic cell cultures was performed using the CalPhos[TM] Mammalian Transfection Kit (Clontech) according to the manufacturer's recommendations. Transfection solution containing plasmid DNA and HEPES-buffered saline were gently mixed, incubated for 20 minutes at room temperature, and added to the cultures. The cultures were incubated in this solution for 4 hours at 37°C and 5% CO_2. Transfection was terminated by removing the transfection solution, washing the monolayers twice with buffered saline glucose (116 mM NaCl, 27.2 mM Na_2HPO_4, 6.1 mM KH_2PO_4, glucose 11.4 mM), and adding fresh cell culture medium to the cells. Immediately after transfection, the cultures were exposed to hypoxia for 24 hours.

Luciferase activity was determined with the Luciferase detection kit (Promega) using a Luminoscan luminometer (Labsystems). After hypoxia, cells were lysed with cell culture lysis buffer and stored at –70°C for further analysis. Lysates from two wells were pooled, centrifuged and 100 μl supernatants from two independent sister cultures were used for each measurement. HIF-1 activity was expressed as the ratio between the relative light units measured with the HIF-1 responsive element (pHBE) and the control pGL3 vector.

Quantitation of tyrosine hydroxylase mRNA level. The TH mRNA level was determined by competitive RT-PCR with an internal RNA standard of the same length and sequence as the target mRNA except for a nine-base mutation in the middle of the fragment. During the amplification, both PCR products were labeled by incorporating biotin- and digoxigenin-labeled primers. Following strand separation, the target and standard amplicons were distinguished by hybridization

Table 1 – Structure of the primers and the capture probes for the target and the internal standard used for the quantitation of tyrosine hydroxylase mRNA levels

Primer and capture probes	Sequences
5' Primer (BIO)	5'-tag-tcc-cca-agg-ttc-atc-3'
3' Primer (DIG)	5'-tac-agc-ccg-aga-caa-gga-3'
CP target	5'-tag-cct-cct-cgg-aac-ctg-gga-acc-cac-tgg-agg-3'
CP internal Standard	5'-tag-cct-cct-cgg-ggg-tga-cac-acc-cac-tgg-agg-3'

Mutations in the internal standard underlined. BIO-biotin labeled, CP-capture probe, DIG - digoxigenin labeled. Fragment length is 216 base pairs. Accession number from the NCBI World Wide Web home page is M 10244.

with specific capture probes immobilized on microtiter plates. Tab. 1 indicates the nucleotide sequences of the primers, the capture probes for the target and the internal standard, as well as the lengths of the amplified fragments. Primer sequences were derived from the TH sequence [15]. The 5'-biotin- and 5'-digoxigenin-labeled primers and the capture probes were from BioTez (Berlin, Germany). The cRNA internal standard contained a stretch of nine mutated bases in the middle of the amplified fragment of the target RNA. The internal standard DNA constructs for TH enabling *in vitro* transcription were generated using the Lig'n scribe kit (Ambion, Austin, TX). Recovery experiments showed the TH mRNA quantitation to be of acceptable accuracy [16]. The correlation coefficient between the theoretical and experimental results was 0.94. The RT-PCR results were linear within the range of 0.05–0.5 μg total RNA. The sensitivity of the method amounts to 400 ag/μg total RNA. The estimated intra-assay variation coefficient was 25 % (n=10).

Dopamine and protein content. DA was quantified by HPLC-EC as reported elsewhere [12]. Briefly, DA was extracted from the monolayers using perchloric acid. Cell precipitates were collected using a rubber policeman and centrifuged. The supernatant was used for DA determination and the pellet dissolved in NAOH and used for total protein determination. Protein determination was carried out by the Bio-Rad D_C Protein Assay according to the manufacturer's (Bio-Rad, Hercules, CA) protocol.

Statistics. The data were analyzed statistically using ANOVA for various groups and across time points followed by a post-hoc Scheffe's test. Difference was considered significant at $p < 0.05$.

Results

The effect of single exposure to hypoxia during the early developmental period on the short-and long-term DA and TH mRNA levels was tested as illustrated in the schematic diagram (Fig. 1). Mesencephalic cell cultures were exposed to hypoxia
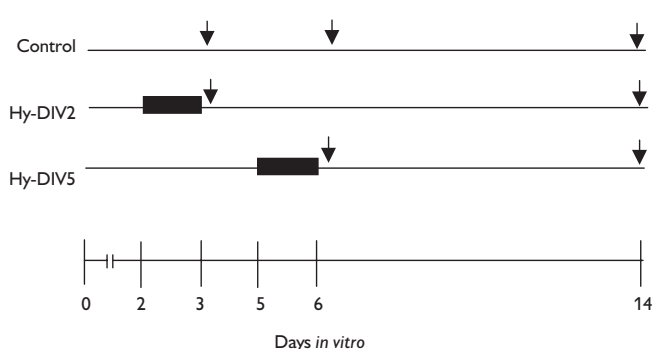

Fig. 1– Schematic diagram showing the design of the experiments. The day of culture preparation was considered as day in vitro zero (DIV 0). Days of sample collection are indicated by arrows. Black rectangles: exposure of cultures to hypoxia (24 h, 10-20 mm Hg oxygen). Hy-DIV2 and Hy-DIV5 indicate exposure to hypoxia on DIV 2 and DIV 5, respectively.

on DIV 2 or DIV 5 for 24 h. To assess short-term hypoxic changes, samples were taken immediately post hypoxia, while long-term changes were estimated in the samples collected 8 – 11 days post hypoxia. Controls were collected on corresponding days (i.e., DIVs 3, 6, and 14).

   The developmental changes in the total protein content per well and dopamine levels of normal cultures correspond to our previous observation [11]. The protein content increased 3-fold between DIVs 3 and 6 and persisted at this level up to DIV 14 indicating normal development (Fig. 2). In accordance with the previous data, DA did not increase between DIVs 3 and 6, but showed a 2-fold increase between DIVs 6 and 14. In contrast, TH mRNA levels increased significantly between DIV 3 and DIV 6, but showed no further changes up to DIV 14.
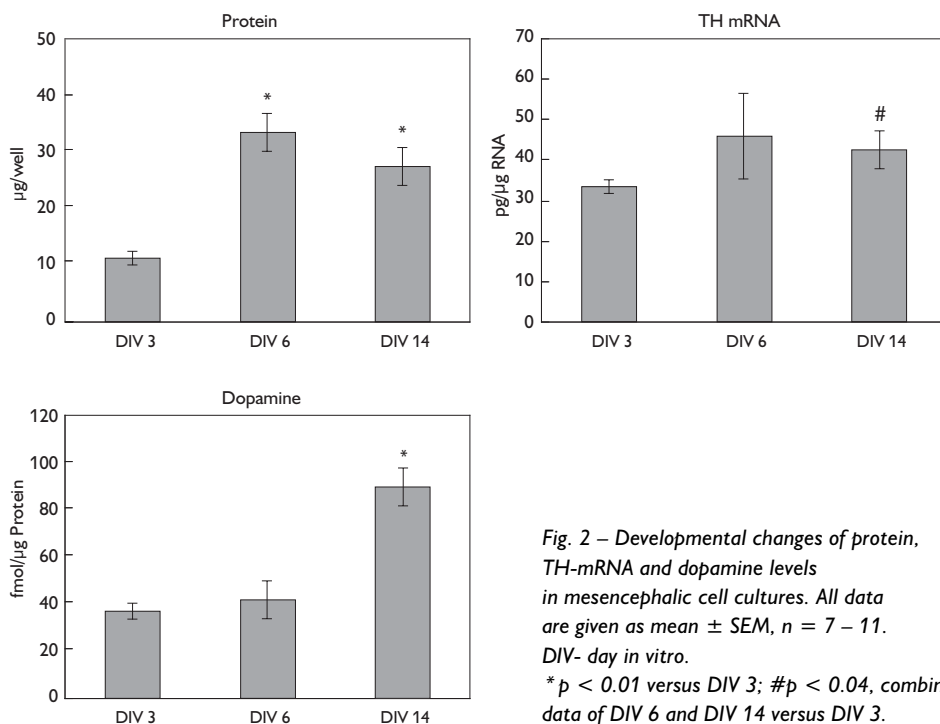

Fig. 2 – Developmental changes of protein,
TH-mRNA and dopamine levels
in mesencephalic cell cultures. All data
are given as mean ± SEM, n = 7 – 11.
DIV- day in vitro.
*p < 0.01 versus DIV 3; #p < 0.04, combined
data of DIV 6 and DIV 14 versus DIV 3.

Table 2 – Hypoxia–induced changes of total protein, TH mRNA and dopamine levels as determined immediately post-hypoxia on DIVs 3 and DIV 6

	Protein	TH mRNA	Dopamine
DIV 3	0.97 ± 0.18	0.88 ± 0.22	1.24 ± 0.14
DIV 6	1.08 ± 0.27	1.14 ± 0.17	0.72 ± 0.12^{x}

Data are given as hypoxia/control ratios. Numbers indicate mean ± SEM, n=7-11. ^{x}p <0.03 versus control.

Experimental cultures were exposed to hypoxia on DIV 2 and DIV 5. Morphological observation by phase contrast microscopy did not reveal any hypoxia-induced cell damage during the entire cultivation period. The exposure of the cell cultures to hypoxia on DIV 2 had no short-term effect on the total protein, TH mRNA, and DA levels (Tab. 2). In contrast, the exposure of 5-day-old cultures to hypoxia reduced the DA content by approximately 30%, while protein
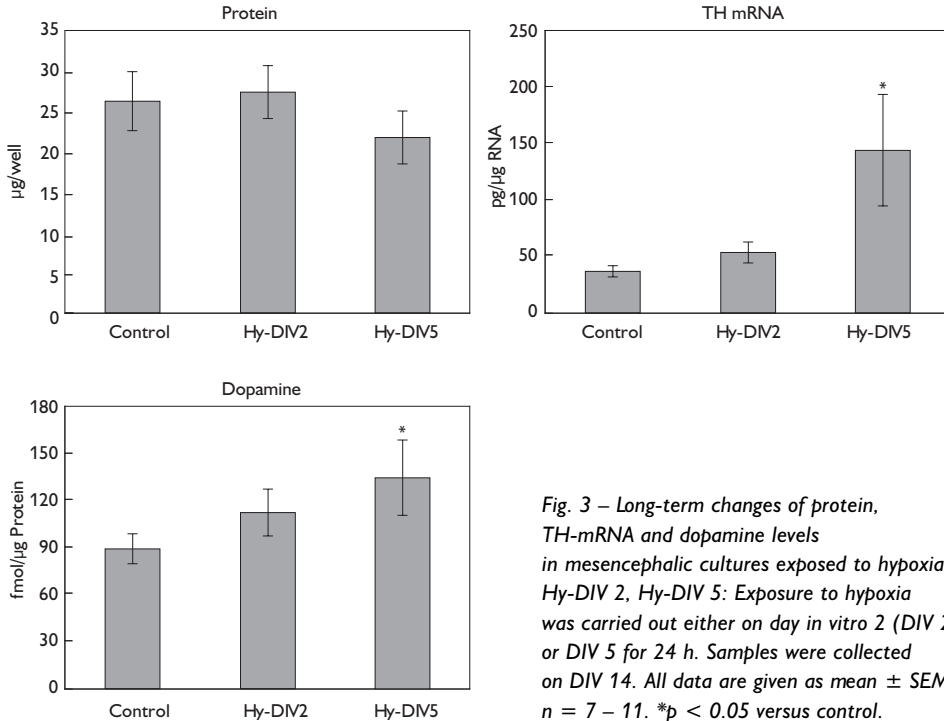

Fig. 3 – Long-term changes of protein, TH-mRNA and dopamine levels in mesencephalic cultures exposed to hypoxia. Hy-DIV 2, Hy-DIV 5: Exposure to hypoxia was carried out either on day in vitro 2 (DIV 2) or DIV 5 for 24 h. Samples were collected on DIV 14. All data are given as mean ± SEM, n = 7 – 11. *p < 0.05 versus control.

Table 3 – Luciferase activity on *in vitro* days 3 and DIV 6 in cultures exposed to hypoxia

Vector	DIV 3		DIV 6	
	Control	Hypoxia (DIV2)	Control	Hypoxia (DIV5)
pGL3	70.2 (12; 115)	43.4 (8.3; 115.3)	2.5 (1; 4.6)[x]	2.8 (0.7; 3.6)
pHBE	82 (14.9; 161.9)	196.1 (23.1; 375.6)	4.6 (1.5; 6.6)[x]	14.1 (2.1; 20.8)
HIF-1	1.5 ± 0.2	6.4 ± 1.0[#]	1.9 ± 0.7	5.0 ± 1.3[#]

Cells were transfected with pGL3 and pHBE. Data for pGL3 and pHBE are given in relative luminometric units measured in monolayers collected from 2 wells. Data were evaluated by nonparametric statistics and given as median (25th; 75th percentiles). Data for HIF-1 are given as mean ± SEM. n = 11 for DIV 3 and n = 5 for DIV 6. [x]p < 0.002 versus DIV 3 (Mann-Whitney U test). [#]p < 0.05 versus control.

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levels and TH mRNA remained unchanged indicating the absence of hypoxia-induced cell damage.

To study the long-term effects of early hypoxia on DA and TH mRNA levels we evaluated these parameters in 14-day-old controls and hypoxia-exposed cultures. Cultures exposed to hypoxia during early development (DIVs 2 and 5) showed a persistent tendency toward long-term dopamine increase. Whereas hypoxia on DIV 2 induced an insignificant 20% increase, DIV 5 hypoxia produced a pronounced increase in TH mRNA and DA levels (3.8- and 1.5-fold, respectively; $p < 0.01$) on DIV 14 (Fig. 3).

To examine a possible correlation between HIF-1 activation during the early developmental period and the long-term increase of TH mRNA levels (DIV 14), we measured HIF-1 activity in sister cultures exposed to hypoxia on DIVs 2 and 5 using the pGL3 reporter system. Tab. 3 shows that hypoxia either on DIV 2 or on DIV 5 did not change the expression of pGL3 in comparison to the controls. However, these hypoxic exposures caused a 3-fold increase in the pHBE/pGL3 ratio indicating similar HIF-1 activation during both developmental periods (Tab. 3). Notably, pGL3-induced luciferase activity in 5-day-old cultures decreased dramatically compared to 2-day-old ones. A similar decrease in transfection efficiency of more mature cells was observed by other authors [17], however, the mechanism of this dramatic decrease is not clear.

Discussion

The major finding of the present study is a pronounced long-term increase in TH mRNA and DA levels in rat mesencephalic neurons caused by a single hypoxic exposure during the putative critical developmental period. The data show that single hypoxia during early development can substantially affect the developmental regulation of TH mRNA transcript and consequently the DA levels.

Whereas the higher steady-state DA level could be explained by increased TH mRNA expression [7], the factors determining the latter phenomenon were not identified. However, some aspects could be considered: (1) A plausible explanation for the hypoxia-induced increase in TH mRNA levels in mesencephalic cultures could be an increase in the number of TH-IR cells. Our previous studies on the same experimental model showed no significant increase in the number of TH-positive neurons [5, 6]. In contrast to our results, Studer *et al.* [18] revealed an increase in the TH-IR cell number caused by oxygen deficiency. However, if compared to the present culture model, these authors used milder hypoxic conditions and mesencephalic cells from 12-day-old embryos, i.e., dopaminergic precursor cells proliferating in response to hypoxia. In our culture model, we used preparations from gestational day 14–15, i.e., more differentiated dopaminergic neurons, which did not proliferate in response to hypoxia. Our results support the suggestion that oxygen levels

may have an effect on catecholaminergic differentiation [19]. (2) Our data show that TH mRNA expression correlates with the hypoxia-induced increase in the DA level. As reported by Kumer and Vrana [7], the activation of TH gene expression by feedback regulation combined with the cross talk between transcriptional factors might mediate the long-term increase in the TH mRNA level. Thus, in our study, the long-term induction of TH mRNA might have been triggered by the acute reduction of intracellular DA level after DIV 5 hypoxia due to hypoxia-stimulated DA release [12]. (3) The stability of mRNA is the other important factor contributing to TH mRNA level. Increased TH mRNA stability following hypoxia (half-life 30 h after hypoxia versus 10 h in the control) was described by Czyzyk-Krzeska *et al.* [20]. However, the 9-day interval between DIV 5 hypoxia and the increase in TH mRNA level revealed on DIV 14 suggests that the prolonged half-life of TH mRNA due to its higher stability can hardly be responsible by itself for the accumulation of the TH transcripts on DIV 14. (4) A well-defined regulatory factor stimulating TH gene expression is HIF-1 [21]. Similar HIF-1 activation in 2- and 5-day-old cultures suggests that HIF-1 is a necessary, but in itself insufficient factor in the long-term induction of TH mRNA. As was shown earlier, hypoxia-induced transcriptional regulation requires a multiprotein complex including hypoxia-inducible factor-1 [22], therefore, other factors could contribute to the observed long-term effects.

Notably, TH mRNA and DA levels were observed to increase at different rates due to early hypoxia and during *in* vitro development. This discrepancy can be explained by the fact that steady-state levels of DA depend not only on the absolute levels of TH mRNA expression and TH mRNA protein levels, but also, more likely, on the balance between synthesis and degradation of DA. It suggests a role of several enzymes in the DA homeostasis regulation [7].

In our previous studies, the putative critical developmental period characterized by hypoxia-induced long-term increase in DA content was restricted to DIVs 1–3 [6]. However, in the present study we showed that DIV 2 hypoxia induced an insignificant increase only, whereas DIV 5 hypoxia resulted in a long-term, statistically significant increase in DA and TH mRNA levels. Therefore, the critical period may be defined not only by the time course of early *in vitro* development, but also by some other, yet unknown factors, for example, temporal changes in the expression of growth factors [23].

Our present data suggest that hypoxia/ischemia during the putative critical developmental period increase TH mRNA expression and the DA content in mesencephalic cell culture. This finding is consistent with our observations in a rat model [24]. Thus, it can be concluded that the increase in TH mRNA levels may play a crucial role in the hypoxia-triggered cascade of reactions affecting the subsequent development of the dopaminergic system of the brain.

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