GENE EXPRESSION OF CATECHOLAMINE SYNTHESIZING ENZYMES IN A5 CELL GROUP AND MODULATION OF TYROSINE HYDROXYLASE mRNA BY IMMOBILIZATION STRESS

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Objective. The A5 group of noradrenergic neurons plays a key role in autonomic mechanisms like cardiovascular regulation, nociception and respiration. The aim of this work was to detect the gene expression of catecholamine synthesizing enzymes in A5 brain nuclei.

Methods. The gene expression of. tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyl-transferase (PNMT) in A5 brain nuclei was estimated. We also investigated various time intervals after the end of the single two-hour immobilization, as well as the effect of short-term repeated immobilization (120 min daily for 7 days) on tyrosine hydroxylase gene expression, the rate-limiting enzymes in catecholamines biosynthesis, in the A5 cell group. For all experiments, reverse transcription with subsequent polymerase chain reaction (RT-PCR) was used.

Results. As expected, we detected a clear signal for TH and DBH mRNA but no signal for PNMT mRNA. Both, single and repeated immobilization stress exposure increased significantly the gene expression of TH in A5 area. Maximal elevation in TH mRNA levels occurred after single immobilization for two hours and subsequent decapitation 24 hours later.

Conclusions. In this study we detected for the first time the presence of DBH mRNA in micro dissected A5 cell group. We also showed how the gene expression of tyrosine hydroxylase changed with the function of time after the single immobilization exposure. Thus, TH mRNA in A5 cell group is modulated by immobilization stress in a time-dependent manner.

Key words: tyrosine hydroxylase – A5 cell group – immobilization stress – gene expression

Stress is a modern civilization factor with a dichotomous function. On the one hand, stress triggers important adaptive functions that promote improved health and survival. On the other hand, excessive stress can be extremely harmful. Catecholamines are among the first molecules to show a response to stressors and are crucial in stimulating action in response to a perceived threat (Sabban and Kvetnansky 2001). Increased levels of catecholamines due to the stress stimuli may reflect the increased gene expression of catecholamine synthesizing enzymes, predominantly tyrosine hydroxylase. Besides the adrenal medulla, which is the main source of catecholamines, stress activates catecholaminergic systems in the brain, which have a widespread influence on neuronal responses to stressors. Using in situ hybridization histochemistry, it was shown that mRNA levels of tyrosine hydroxylase (TH) were

increased by a single and long-term repeated immobilization in the locus coeruleus, A1, A2 and A5 noradrenergic cell groups of the brain (Rusnak et al. 2001). While the A1 and A2 noradrenergic neurons project to hypothalamic and limbic areas, axons from the A5 neurons descend to the spinal cord and innervate the sympathetic preganglionic neurons in the intermediolateral cell column. The locus coeruleus provides both ascending (mainly to the forebrain) and descending (to the spinal cord) noradrenergic fibers (for review see Palkovits 1999). It has been shown by electrophysiological and pharmacological studies (Byrum and Guynet 1987; Clark and Proudfit 1993; Huangfu and Guyenet 1997; Schreihofer and Guyenet 2000) that A5 neurons are involved in the control of cardiovascular and sympathoadrenal activities.

The aim of this work was to study the gene expression of catecholamine synthesizing enzymes in the A5 brain cell group using sensitive reverse transcription with subsequent polymerase chain reaction (RT-PCR). It is already known that immobilization stress elevates the TH mRNA levels three hours after the end of single exposure (Rusnak et al. 2001), therefore we want to extend the current knowledge on how the gene expression of this enzyme is changed with the function of time. Thus, we studied TH mRNA levels in different time periods after the end of the single immobilization stress exposure. We also investigated the effect of repeated immobilization for short period (7-times) on TH gene expression in the A5 cell group.

Materials and Methods

Animals and immobilization. Male Sprague-Dawley rats (280-320g) approximately three month old obtained from Suzfeld, Germany were used in all experiments. Animals were housed four per cage and maintained under control conditions (22 ± 2°C, 12 h light/dark cycle, light on at 6:00 a.m.). Food and water were available *ad libitum*. All experiments with animals were approved by the Ethic Committee of the Institute of Experimental Endocrinology.

Immobilization stress was performed as described previously (Kvetnansky and Mikulaj 1970). In the process of a single immobilization, rats were subjected to immobilization once for 120 minutes and decapitated immediately, 3 hours and 24 hours after the termination of immobilization stimulus. Control rats were sacrificed immediately after the removal from their home cages. Repeated stress was achieved by immobilizing animals for 7 days, 2 hours daily. Decapitation followed three hours after the last immobilization. As so-called „adapted control" group we used a group of rats immobilized for 6 days, 2 hours daily and decapitated 24 hours after the last immobilization (n=4).

The A5 brain area was dissected bilaterally by micropunch technique (Palkovits 1973) according to the punching guide atlas of Palkovits and Brownstein (1988). Briefly, after a quick removal, brains were immediately frozen on dry ice and cut into 300 μm thick serial coronal sections in a cryostat (Reichert) at –12°C. The A5 cell group was isolated under the dissection microscope by special metal punching needles with inner diameter of 500 μm. Finally, the tissue pellets were stored at -70°C for the later analysis.

Isolation of RNA and relative quantification of mRNA levels by RT-PCR. Total RNA was isolated from frozen A5 tissue samples by RNAzol™ (Tel-Test, USA). Reverse transcription was performed from 5 μl of total RNA using Ready-To-Go You-Prime First-Strand Beads (AP Biotech) and $pd(N)_6$ primer according to manufacturer protocol.

The determination of catecholamine synthesizing enzymes, tyrosine hydroxylase, dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) as well as housekeeper glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was carried out by RT-PCR. Specific primers, annealing temperatures, number of cycles and size of each fragment for TH, DBH, PNMT and GAPDH mRNAs are shown in Table 1.

As a control for semiquantitative evaluation of PCR, gene expression of housekeeper GAPDH was used. As a positive control, mRNA of adrenal medulla was used in each RT-PCR. PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide. Intensity of individual bands was evaluated by Kodak Camera and PCBAS 2.08e software.

Statistical evaluation. Results are presented as mean ± S.E.M. and each value represents an average of four animals. Statistical differences among groups were evaluated by one-way analysis of variance

Table 1
Oligonucleotide sequences of primers used in PCR for amplification of specific fragments of TH, DBH, PNMT and GAPDH mRNA, together with optimal annealing temperatures, sizes (in bp) and number of cycles

Name of the gene	Sequences of primers	Temp. of annealing	No. of cycles	Size of fragment	Reference
TH	**THR1**: 5'-GAA GGG CCT CTA TGC TAC CCA-3'				
	THR2: 5'-TGG GCG CTG GAT ACG AGA-3'	63°C/1'	35	645 bp	
DBH	**DBHR1**: 5'-GAC TCA ACT ACT GCC GGC ACG T-3'				
	DBHR2: 5'-CTG GGT GCA CTT GTC TGT GCA GT-3'	60°C/1'	35	320 bp	
PNMT	**PTR$_S$1**: 5'-CCG ATG AGA AGG AGA TGA CC-3'				
	PTR$_S$2: 5'-CTA CCT CCG CAA CAA CTA CG-3'	56°C/1'	35	543 bp	Comer AM et al. 1997
GAPDH	**GPH1**: 5'-AGA TCC ACA ACG GAT ACA TT-3'				
	GPH2: 5'-TCC CTC AAG ATT GTC AGC AA-3'	60°C/1'	30	309 bp	Lou YK et al. 1995

(ANOVA). Values of $p<0.05$ were considered to be significant. For multiple comparisons, an adjusted t-test with p values corrected by Bonferroni method was used (Instat, GraphPad Software, USA).

Results

Identification of the TH, DBH and PNMT gene expression in A5 brain area. In the A5 cell group of rat brain, gene expression of TH, DBH and PNMT was determined (Figure 1). As a positive control, the adrenal medulla (AM) was used. As expected, after RT-PCR we detected a clear signal of 645 bp for TH mRNA and of 320 bp for DBH mRNA, which fully corresponds to the size of amplified fragments from the adrenal medulla. Nevertheless, no signal was observed for PNMT mRNA from A5 region, although in the AM a clear signal was shown.

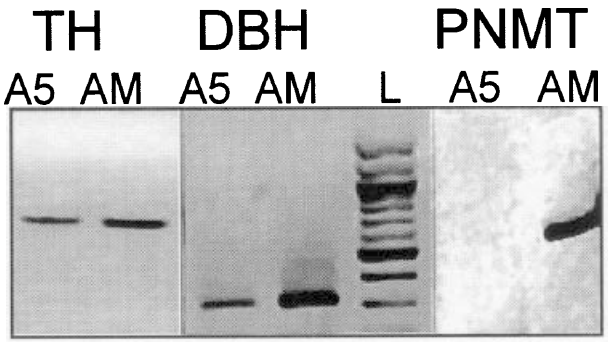

Fig. 1 Determination of tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) mRNA levels in A5 area of rat brain. As a positive control, adrenal medulla (AM) was used. In A5 area (A5), a clear signal for TH (645 bp fragment) and DBH mRNA (320 bp fragment) identical with that in AM was observed. PNMT mRNA was not detected in A5 region, although a positive control of AM revealed a clear 543 bp fragment of PNMT mRNA (L=base pair ladder).

Effect of single immobilization stress on the TH gene expression in A5 cell group of rat brain is time-dependent. Various time intervals after single immobilization stress on tyrosine hydroxylase mRNA levels in A5 area of rat brain were examined. Animals were immobilized one-time for 120 min and decapitated immediately, 3 hours or 24 hours after termination of the stress stimulus (Figure 2). After a single immobilization for two hours, the significant increase in TH mRNA levels in all immobilized groups of rats was found compared to unstressed control rats. Exposure of rats to single immobilization for two hours with immediate decapitation produced about 2-fold elevation of TH mRNA levels, 3 hours after termination of the stress stimulus the elevation of TH mRNA was about 1.5-2.0-fold. The marked elevation, about 3-fold, compared to the control group occurred in a group of rats one-time immobilized for 2 hours and decapitated 24 hours later. After 24 hours, further increase (1.6-fold, $p<0.05$) in TH mRNA levels was found compared to TH mRNA observed 3 hours after the end of stressful stimulus.

Effect of seven-times repeated immobilization stress on TH gene expression in A5 cell group of rat brain. After both single and repeated immobilization, there was significant increase of TH mRNA levels compared to control, unstressed rats (Figure 3). Exposure of rats to single immobilization pro-

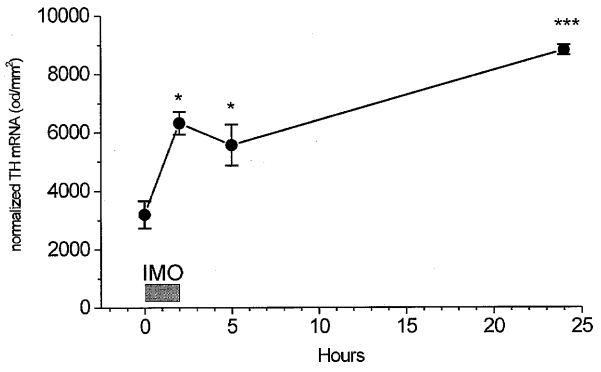

Fig. 2 Effect of single immobilization stress (IMO, 120 minutes) on tyrosine hydroxylase mRNA levels in A5 area of rats decapitated immediately, 3 hours and 24 hours after immobilization termination. Results are normalized relatively to the housekeeper GAPDH. Values are displayed as mean ± S.E.M. and each value represents an average of four animals. Statistical significance was calculated as described in Material and Methods and shows the difference between the unstressed control and immobilized animals. * represents P<0.05, *** P<0.001.

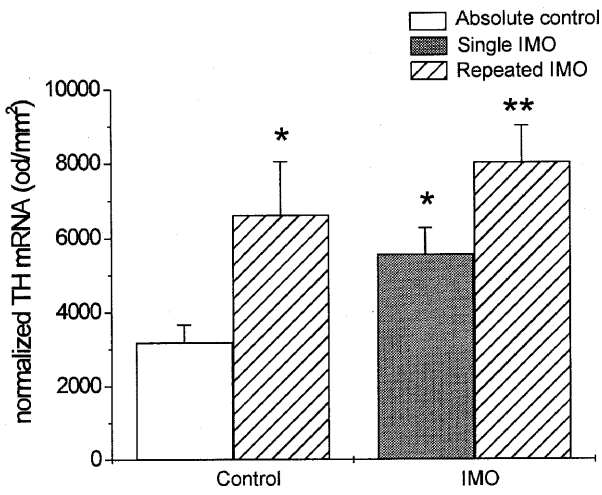

Fig. 3 The effect of single (filled column) and seven-times repeated (striped column) immobilization stress on tyrosine hydroxylase mRNA levels in A5 brain area of rats. Empty column represents the absolute control group and striped control column represents an „adapted control" group of rats immobilized for six-times and decapitated 24 hours after the last immobilization. Results are normalized relatively to the housekeeper GAPDH. Values are displayed as mean ± S.E.M. and each value represents an average of four animals. Statistical significance was calculated as described in Material and Methods and shows the difference between the unstressed control and immobilized animals. * represents P<0.05, ** P<0.01.

duced about 1.5-2-fold elevation and seven-times repeated immobilization about 2.5-fold elevation of TH mRNA levels. In adapted control group of rats (immobilized for 6 days two hours daily) was increased approximately twice compared to the absolute control. No significant changes in TH mRNA levels were observed between adapted control and seven-times repeated immobilization.

Discussion

Since the first description of the A5 cell group (Dahlstrom and Fuxe 1964) several studies confirmed that these brainstem neurons are noradrenergic and project to the spinal cord. Thus, it is not surprising that A5 area expresses TH and DBH mRNAs, but not PNMT. Although TH mRNA was already demonstrated in this area by in situ hybridization histochemistry (Rusnak et al. 2001), we are the first who detected the presence of DBH mRNA in microdissected A5 cell group. Since TH is a rate-limiting step in the catecholaminergic synthesis, modulation of this enzyme's mRNA, protein levels, or activity would affect the catecholamine production in this area. Our results clearly show that both, single and repeated immobilization stress increased gene expression of the TH mRNA in A5 area. These findings are in a good agreement with in situ hybridization histochemistry results on elevation of TH mRNA in the A5 cell group after the single and 42-times repeated immobilization exposure (Rusnak et al. 2001). After single two-hour immobilization the mRNA levels of TH further increase up to 24 hours in A5 cell group. Immobilization induced increase in TH mRNA has been observed in adrenal medulla (Mcmahon et al. 1992; Nankova et al. 1994), as well as in the locus coeruleus (Rusnak et al. 1998; Rusnak et al. 2001), etc. In adrenal medulla, the response to stressors is rapid, especially for TH, where one episode of immobilization is sufficient for maximal elevation of TH mRNA (Nankova et al. 1994; Sabban and Kvetnansky 2001). On the other site, sympathetic ganglia respond more slowly and repeated episodes of immobilization stress are required to induce a maximal elevation in TH mRNA level (Nankova et al. 1996). In A5 area, TH mRNA level was

increased immediately after two hours immobilization, although it did not reach the maximal levels. Maximal elevation in TH mRNA levels occurred after single immobilization for 2 hours and subsequent decapitation 24 hours later. Adrenal medullary TH mRNA levels were found in that time interval back at control values (Nankova et al. 1994; Sabban and Kvetnansky 2001).

Other brainstem norepinephrine neurons (A1, A2, locus coeruleus) are also activated by stressful stimuli, resulting in an increased release of norepinephrine in the hypothalamus and leading to the activation of hypothalamic-pituitary-adrenocortical axis (Pacak et al. 1995; Rusnak et al. 2001). Since A5 noradrenergic neurons play a key role in autonomic mechanisms, like cardiovascular regulations, nociception, and respiration (Huangfu and Guyenet 1997; Clark and Proudfit 1993), involvement of this area in regulation of the sympathoadrenal system activation during stress is very probable. However, this function of the A5 cell group in stress responses has to be elucidated.

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References

Byrum CE, Guyenet PG: Afferent and efferent connections of the A5 noradrenergic cell group in the rat. J Comp Neurol **261**, 529-542, 1987

Clark FM, Proudfit HK: The projections of noradrenergic neurons in the A5 catecholamine cell group to the spinal cord in the rat: anatomical evidence that A5 neurons modulate nociception. Brain Res **616**, 200-210, 1993

Comer AM, Yip S, Lipski J: Detection of weakly expressed genes in the rostral ventrolateral medulla of the rat using micropunch and reverse transcription-polymerase chain reaction techniques. Clin Exp Pharmacol Physiol **24**, 755-759, 1997

Dahlstrom A, Fuxe K: Localization of monoamines in the lower brain stem. Experientia **20**, 398-399, 1964

Huangfu D, Guyenet PG: Autoactivity of A5 neurons: role of subthreshold oscillations and persistent Na^+ current. Am J Physiol **273**, H2280-9, 1997

Kvetnansky R, Mikulaj L: Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. Endocrinology **87**, 738-741, 1970

Lou YK, Liu DT, Whitworth JA, Morris BJ: Renin mRNA, quantified by polymerase chain reaction, in renal hypertensive rat tissues. Hypertension **26**, 656-664, 1995

Mcmahon A, Kvetnansky R, Fukuhara K, Weise VK, Kopin IJ, Sabban EL: Regulation of tyrosine hydroxylase and dopamine beta-hydroxylase mRNA levels in rat adrenals by a single and repeated immobilization stress. J Neurochem **58**, 2124-30, 1992

Nankova B, Kvetnansky R, Mcmahon A, Viskupic E, Higemagalur B, Frankle G, Fukuhara K, Kopin IJ, Sabban EL: Induction of tyrosine hydroxylase gene expression by a nonneuronal nonpituitary-mediated mechanism in immobilization stress. Proc Natl Acad Sci **91**, 5937-41, 1994

Nankova B, Kvetnansky R, Hiremagalur B, Sabban B, Rusnak M, Sabban EL: Immobilization stress elevates gene expression for catecholamine biosynthetic enzymes and some neuropeptides in rat sympathetic ganglia: effects of adrenocorticotropin and glucocorticoids. Endocrinology **137**, 5597-5604, 1996

Pacak K, Palkovits M, Kopin IJ, Goldstein DS: Stress-induced norepinephrine release in the hypothalamic paraventricular nucleus and pituitary-adrenocortical and sympathoadrenal activity: In vivo microdialysis study. Front Neuroendocrinol **16**, 89-150, 1995

Palkovits M: Isolated removal of hypothalamic or other brain nuclei of the rat. Brain Res **59**, 449-450, 1973

Palkovits M, Brownstein MJ: Maps and guide to microdissection of the rat brain. Elsevier Science Publishing Co., New York, 1988

Palkovits M: Interconnections between the neuroendocrine hypothalamus and the central autonomic system. Front Neuroendocrinol **20**, 270-295, 1999

Rusnak M, Zorad S, Buckendahl P, Sabban EL, Kvetnansky R: Tyrosine hydroxylase mRNA levels in locus ceruleus of rats during adaptation to long-term immobilization stress exposure. Mol Chem Neuropathol **33**, 249-58,1998

Rusnak M, Kvetnansky R, Jelokova J, Palkovits M: Effect of novel stressors on gene expression of tyrosine hydroxylase and monoamine transporters in brainstem noradrenergic neurons of long-term repeatedly immobilized rats. Brain Res **899**, 20-35, 2001

SABBAN EL AND KVETNANSKY R: Stress-triggered activation of gene expression in catecholaminergic systems: dynamics of transcriptional events. TINS **24**, 91-98, 2001

SCHREIHOFER AM AND GUYENET PG: Sympathetic reflexes after depletion of bulbospinal catecholaminergic neurons with anti-D?H-saporin. Am J Physiol Regulatory Integrative Comp Physiol **279**, R729-R742, 2000

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