COMPARATIVE STUDY OF CATECHOLAMINE SYNTHESIZING ENZYMES IN ADRENAL MEDULLA OF CRH KNOCK-OUT MICE, THEIR CRH (+/+) MATES AND SPRAGUE-DAWLEY RATS

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Objective. Corticotropin-releasing hormone deficient mice (CRH-KO) serve as an interesting model to understand the role of CRH in the regulation of adrenomedullary system. The aim of this study was to compare tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT) on the levels of gene expression and protein in adrenal medulla of CRH-KO mice, their CRH (+/+) mates and Sprague-Dawley (SD) rats.

Methods. Levels of TH and PNMT mRNA were determined by reverse transcription with subsequent polymerase chain reaction (RT-PCR) and quantified relatively to the housekeeper glyceraldehyde-3-phosphate dehydrogenase. The amount of TH and PNMT protein was determined by Western blot analysis and visualized by enhanced chemiluminiscence.

Results. We detected a clear signal of 645 bp for TH mRNA and of 260 bp for PNMT mRNA in adrenal medulla of rats and CRH (+/+) mice, with higher concentration of TH and PNMT mRNA in rat adrenal medulla. Subsequently, TH and PNMT immunoprotein was measured and we found significantly higher amount of TH and also PNMT protein in the rat compared to CRH (+/+) mice. On the other hand, the amount of TH and PNMT immunoprotein in adrenal medulla of CRH-KO mice was significantly lower compared to CRH (+/+) mice.

Conclusions. Our results indicate the lower production of adrenomedullary TH and PNMT protein in CRH (+/+) mice compared to rats, which reflects the lower gene expression of these enzymes in adrenal medulla of mice. We also demonstrated the differences in TH and PNMT protein levels between CRH (+/+) and CRH-KO (-/-) mice.

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Key words: Corticotropin-releasing hormone – CRH knock-out mice – Adrenal medulla – Tyrosine hydroxylase – Phenylethanolamine-N-methyltransferase

The hypotalamo-pituitary-adrenal (HPA) axis and the sympathetic nervous system are anatomically and functionally interconnected and they are coordinately activated in response to stressful stimuli. A major hypothalamic activator of the HPA axis is corticotropin-releasing hormone (Vale et al. 1983). This 41 amino-acid neuropeptide, produced in the paraventricular nucleus and in many regions of the cerebral cortex is thought to play a role in activation of catecholamine secretion directly via interaction between CRH neurons in the hypothalamus and catecholaminergic neurons in the brainstem (Majzoub et al. 1996). Indirect modulation occurs via the secretion of adrenocorticotropic hormone (ACTH) by the pituitary. The secretion of ACTH is the primary stimulus for glucocorticoid release.

The gene expression of adrenomedullary tyrosine hydroxylase (TH), the rate-limiting enzyme that catalyses the first step of catecholamine biosynthesis, is not under the transcriptional regulation of gluco-

corticoids and thus also of the glucocorticoid-responsive element, but under the control of cAMP responsive element and AP1 domain (Sabban and Kvetnansky 2001) and of cholinergic sympathetic input from splanchnic nerves (Nagatsu et al. 1991; Kilbourne et al. 1992; Hiremagalur et al. 1993; Vietor et al. 1996; Jahng et al. 1997). On the contrary, phenylethanolamine N-methyltransferase (PNMT), the enzyme that catalyses the final step in the catecholamine biosynthetic pathway converting norepinephrine to epinephrine, is influenced by glucocorticoids (Viskupic et al. 1994; Krizanova et al. 2001; Sabban and Kvetnansky 2001). The latter regulate PNMT in two ways: posttranscriptionally via regulation of PNMT methyl donor a co-substrate, S-adenosylmethionine, and via regulation of PNMT gene expression.

CRH knock-out mice can serve as a suitable model for studying the mechanisms involved in the functional relation between adrenocortical and adrenomedullary systems. Due to a targeted disruption of the CRH gene, CRH-KO mice exhibit marked CRH and also glucocorticoid deficiency. In such mice, zona fasciculata which is primarily responsible for corticosterone production, appears mostly atrophic, but in contrast to such atrophic appearance the histology of adrenal medulla appears normal (Muglia et al. 1995). Nevertheless, it was already demonstrated that CRH deficiency in CRH-KO mice results in impaired PNMT gene expression and also in lowering epinephrine synthesis and secretion in the adrenal medulla, possibly due to impaired adrenocortical corticosterone production (Jeong et al. 2000).

For better understanding of the physiological significance and relative contribution of CRH and glucocorticoids to the regulation of adrenomedullary catecholaminergic system, in this work we focused on comparison of the tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT) on the levels of gene expression and protein in adrenal medulla (AM) of CRH-KO (-/-) mice, their (+/+) littermates and Sprague-Dawley (SD) rats.

Material and Methods

Animals. Male Sprague-Dawley rats (280-320g) obtained from Suzfeld, Germany and male CRH (+/+) and CRH knock-out mice (20-25g) bred in our facility were used in this comparative study. CRH knock-out line was originally obtained from Harvard Medical School, Department of Endocrinology, Boston, USA. Animals were maintained under controlled environmental conditions (22±1^{0}C, 12 h light/dark cycle, light on at 06:00 a.m.). Food and water were available ad libitum. The Ethic Committee of the Institute of Experimental Endocrinology approved all experimental procedures with animals. Rats and mice were sacrificed immediately after removal from their home cages and adrenal medullas were isolated, frozen in liquid nitrogen and stored at -70^{0}C for the further analysis.

Isolation of RNA and semiquantitative determination of mRNA levels by RT-PCR. Total RNA was isolated from frozen adrenal medullas by RNAzol^{TM}(Tel-Test, USA). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Beads (AP, Biotech) and pd(N)$_6$ primer according to manufacturer's protocol. PCR for TH and PNMT was carried out using the following primers: THR1 5'-GAA GGG CCT CTA TGC TAC CCA-3', THR2 5'-TGG, GCG CTG GAT ACG AGA-3', PT1 5'-TAC CTC CGC AAC AAC TAC GC-3' and PT2 5'-AAG GCT CCT GGT TCC TCT CG-3', giving 645 bp (TH) and 260 bp (PNMT) fragment. After the initial denaturation at 94^{0}C for 5 min, PCR for TH included 35 cycles of denaturation at 94^{0}C for 1 min, annealing at 63^{0}C for 30 sec and polymerization at 72^{0}C for 1 min. For PCR of the PNMT, the annealing temperature was 60^{0}C, other conditions remained the same as are for TH cDNA. For semiquantitative evaluation of PCR, primers for the housekeeper glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GPH1 5'-AGA TCC ACA ACG GAT ACA TT-3' and GPH2 5'-TCC CTC AAG ATT GTC AGC AA-3') were used to amplify 309 bp fragment. PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide. Intensity of individual bands was evaluated by analysis system STS 6220I (Ultra-Lum, Inc.) and PCBAS 2.08e software.

Western blot analysis. Adrenal medullas were homogenized in 0.05M potassium phosphate, pH 6.65/0.2%Triton X-100 and centrifuged at 10.000 x g for 20 min at 4^{0}C. The protein concentration was measured according to Bradford (Bradford 1976). Afterwards, supernatant proteins were fractionated

by 10% SDS-PAGE gel electrophoresis and transferred to the supported nitrocellulose membranes (Hybond™C Extra, AP Biotech) using semidry electrophoretic blotting system (EBU-6000, C.B.S.Scientific company Inc.). Non-specific binding sites were blocked by immersing the membranes in 5% dry non-fat milk in Tris-buffered saline Tween (TBST) for one hour at the room temperature on a shaker. All subsequent washes and primary/secondary antibody incubations were also carried in TBST. Levels of the tyrosine hydroxylase immunoreactive protein were determined using a monoclonal primary antibody against mouse TH (clone 2/40/15, dilution 1:5000, Boehringer Mannheim Biochemica) and levels of phenylethanolamine N-methyltransferase immunoprotein were assigned by using a polyclonal primary antibody against bovine PNMT (dilution 1:1000, Protos Biotech). This antiserum crossreacts with PNMT in human, monkey, rodents and cats. HRP labeled secondary antibodies (dilution 1:5000, Amersham Life Sciences) were visualized by Western light chemiluminiscent detection system (ECL, AP Biotech). The optical density of individual bands was detected by PCBAS 2.08e software.

Statistical evaluation. Data are presented as mean±S.E.M. and each value represents an average of 5 measurements. Statistical differences among strains were determined by one-way analysis of variance (ANOVA). Values of $P<0.05$ were considered to be significant.

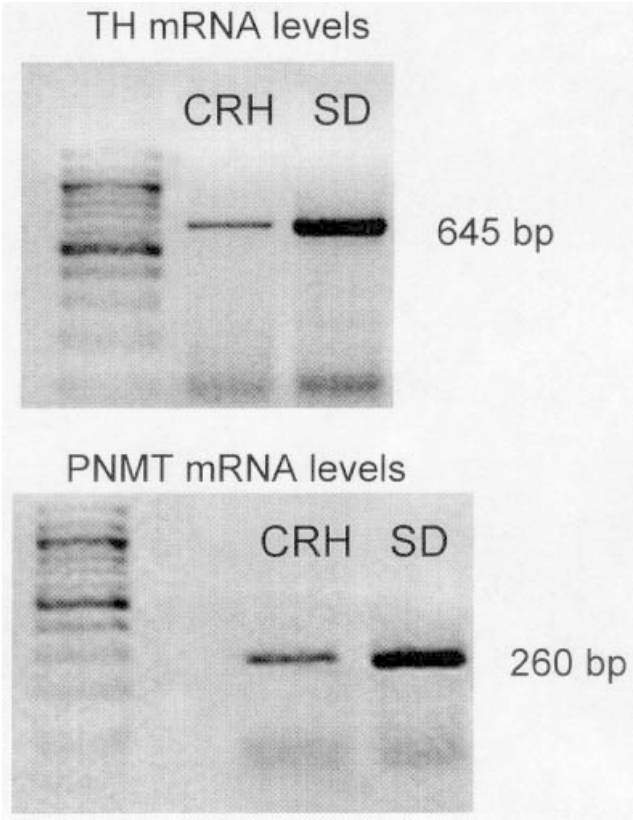

Fig 1 Identification of tyrosine hydroxylase (TH) and phenylethanolamine N-methyltranferase (PNMT) mRNA in adrenal medulla of Sprague-Dawley rat (SD) and CRH (+/+) mouse. The clear signal for TH (645 bp fragment) and PNMT (260 bp fragment) was observed.

Results

TH and PNMT gene expression and immunoprotein levels in adrenal medulla of Sprague-Dawley rats and CRH (+/+) mice. To compare adrenomedullary TH and PNMT gene expression in rats and mice the levels of TH and PNMT mRNA were analyzed. After RT-PCR we detected a clear signal of 645 bp for TH mRNA and of 260 bp for PNMT mRNA in both, rats and mice (Figure 1). TH and PNMT mRNA levels were quantified relatively to the housekeeper GAPDH. Gene expression of both, TH and PNMT was approximately 2.5-times lower in CRH (+/+) mice compared to SD rats. Similar results were observed when we compared the protein levels of TH and PNMT in AM of rats and CRH (+/+) mice (Figure 2). In order to quantify the ratio of each enzyme tested in AM of rats and mice precisely, we loaded different concentrations of total proteins (in the range 2.5 –5.0 mg) on the gel. In CRH (+/+) mice AM, both TH and PNMT proteins were approximately 1.4-times less abundant compared to rat AM.

Comparison of TH and PNMT immunoprotein levels in adrenal medulla of CRH-KO (-/-) and CRH (+/+) mice. To compare the amount of TH and PNMT immunoreactive protein in adrenal medulla of CRH-KO and CRH (+/+) mice, we performed the Western blot analysis with subsequent ECL detection. We compared TH and PNMT immunoprotein levels in AM of CRH (+/+), CRH (+/-) and CRH (-/-) mice in the range of 2.5-25.0 mg of total protein loaded. We revealed no significant difference in TH immunoprotein levels between CRH (+/+) homozygote and CRH (+/-) heterozygote (Figure 3). Neverthe-

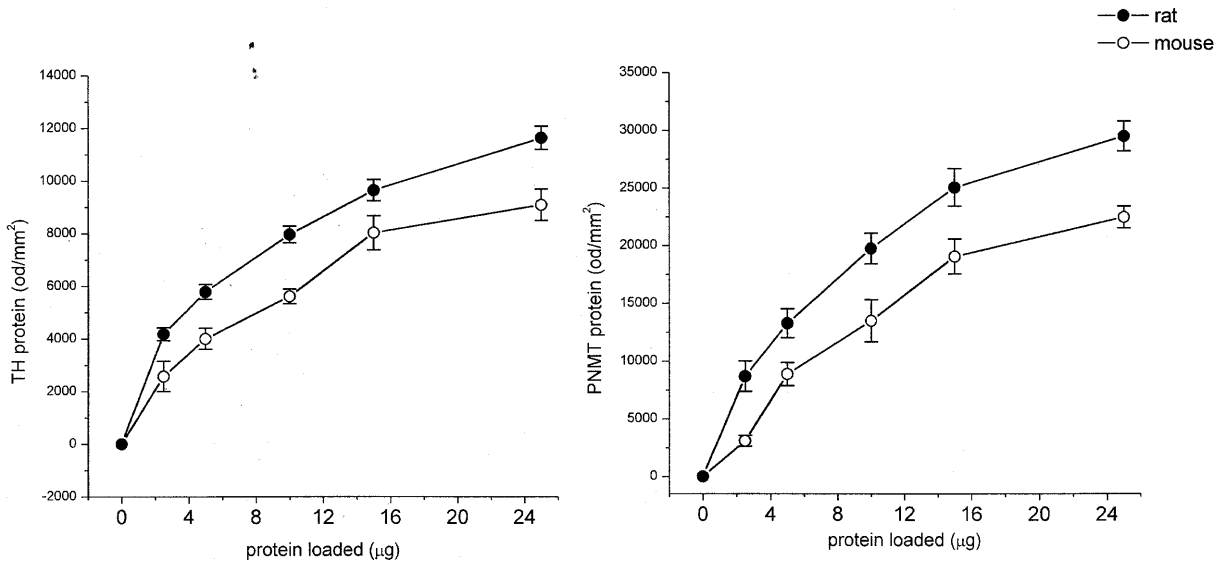

Fig 2 Comparison of TH (left) and PNMT (right) protein levels in adrenal medulla of SD rats (filled circles) and CRH (+/+) mice (empty circles). Levels of both, TH and PNMT proteins were significantly higher in the adrenal medulla of rats compared to mice. Results are displayed as mean ± S.E.M. and each value represents an average of 5 measurements.

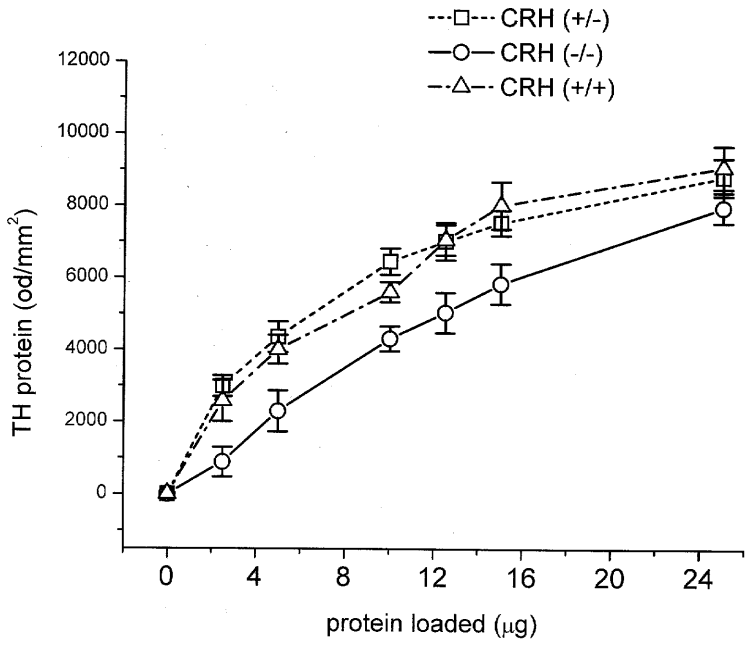

Fig 3 Comparison of the TH immunoprotein levels in adrenal medulla of CRH (+/+), CRH (+/-) and CRH (-/-) mice. Any significant difference in TH immunoprotein levels was observed between CRH (+/+) and CRH (+/-) mice. In the contrary, the amount of TH protein was significantly lower ($p<0.05$) in adrenal medulla of CRH (-/-) mice compared to their not knock-out littermates. Values are displayed as mean + S.E.M. and each value represents an average of 5 measurements.

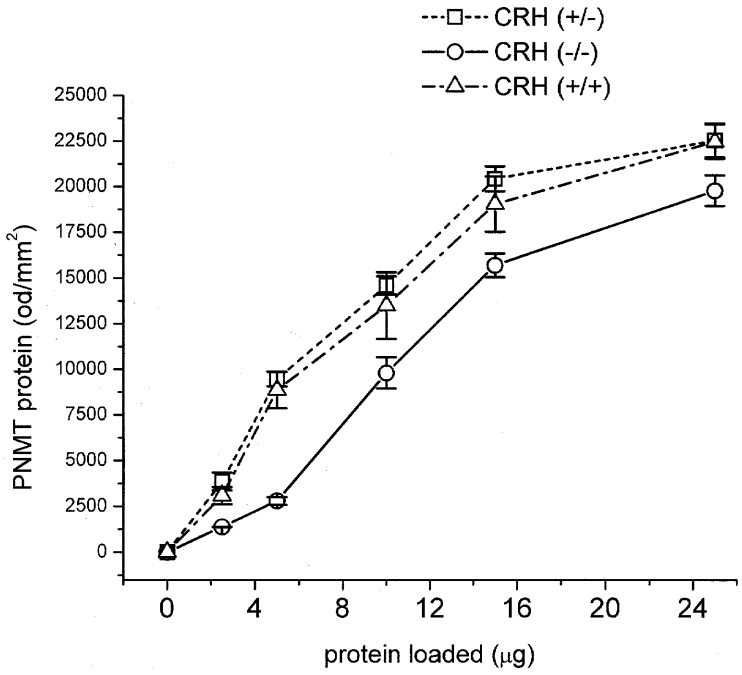

Fig 4 Comparison of the PNMT immunoprotein levels in adrenal medulla of CRH (+/+), CRH (+/-) and CRH (-/-) mice. Any significant difference in PNMT immunoprotein levels was observed between CRH (+/+) and CRH (+/-) mice. Nevertheless, the amount of PNMT protein was significantly decreased ($p<0.05$) in adrenal medulla of CRH (-/-) mice compared to their not knock-out littermates. Values are displayed as mean + S.E.M. and each value represents an average of 5 measurements.

less, the amount of TH immunoreactive protein in adrenal medulla of CRH-KO mice was significantly lower compared to CRH (+/+) homozygote and/or heterozygote (Figure 3). PNMT immunoprotein levels correspond nicely with our results on TH immunoprotein levels (Figure 4). There was no significant difference between CRH (+/+) homozygote and CRH (+/-) heterozygote, but the amount of PNMT immunoreactive protein in adrenal medulla of CRH-KO mice was significantly lower compared to CRH homozygote/heterozygote (Figure 4).

Discussion

In this study we demonstrated significant differences in the adrenomedullary TH and PNMT mRNA levels between Sprague-Dawley rats and CRH (+/+) mice. We found that basal TH and also PNMT mRNA levels in rats are significantly higher than these in mice. Subsequently, the measurements of adrenomedullary TH and PNMT immunoprotein levels revealed the similar results. Although, to our knowledge there is no information about the differences in catecholamine synthesizing enzymes gene expression and protein levels in adrenal medulla of rats and mice, our results clearly show that CRH (+/+) mice express lower amounts of both TH and PNMT as compared to SD rats. Nevertheless, physiological relevance of this observation remains to be elucidated.

In our study we also compared CRH-KO and CRH (+/+) mice. We found the difference in adrenomedullary TH immunoprotein level between CRH-KO and CRH (+/+) mice. The amount of basal TH protein was significantly lower in CRH-KO mice compared to CRH (+/+) mice. Jeong et al. (2000) found the similar basal and restraint-induced TH mRNA levels in both genotypes, CRH-KO and CRH (+/+) mice. Thus, the regulation of TH gene expression, protein and activity remains to be elucidated.

CRH is thought to be important for the function of the adrenomedullary catecholaminergic system, both via actions within the brain as well as by activation of the HPA axis (Jeong et al. 2000). Deafferentation of medial hypothalamus diminishes the im-

mobilization-induced PNMT activity (Kvetnansky et al. 1995) and central CRH antagonist administration blocks the increase in plasma epinephrine induced by central CRH injection in rats, suggesting a specific role for CRH in adrenal epinephrine synthesis (Jeong et al. 2000). In addition, CRH mRNA (Thompson et al. 1987) and peptide (Udelsman et al. 1986; Bruhn et al. 1987) were found in adrenal medulla. This local synthesis of CRH can play a role in regulation of catecholamines.

ACTH and glucocorticoids have been also suggested to regulate both central and peripheral catecholaminergic system (Jeong et al. 2000). Hypophysectomy prevented the immobilization-induced increase in adrenal PNMT gene expression (Viskupic et al. 1994; Kvetnansky et al. 1995; Krizanova et al. 2001). Partial restoration of adrenal PNMT mRNA levels following ACTH treatment of hypophysectomized rats has been also reported (Kvetnansky et al. 1995). Jiang et al. (1989) also demonstrated that hypophysectomy-induced depletion of corticosterone causes blunted adrenal PNMT gene expression and activity in rats. Kvetnansky et al. (1995) described significantly elevated PNMT mRNA levels and the tendency of TH mRNA for an increase in repeatedly treated rats with cortisol. It is well known that glucocorticoids may act through GREs in the PNMT gene promoter region to induce PNMT expression and activity (Jeong et al. 2000). Taken together, all these observations suggest that CRH-ACTH-glucocorticoids cascade can influence the catecholaminergic system in adrenal medulla. Our results revealed that the amount of basal TH and also PNMT immunoprotein in CRH-KO mice is significantly lower compared to CRH (+/+) mice, despite the normal size and histologic appearance of adrenal medulla (Muglia et al. 1995). CRH-KO mice exhibit significantly lower plasma epinephrine, though higher plasma norepinephrine, when evaluated in the basal conditions (Muglia et al. 2000). Jeong et al. (2000) also described the lower basal and restraint-induced adrenal PNMT mRNA and enzyme activity levels in CRH-KO than those in CRH (+/+) mice. Based on our results and in accordance with previous reports, we supposed that CRH deficiency in CRH-KO mice leads to impaired PNMT gene expression, which can result in lower PNMT immunoprotein level compared to CRH (+/+) mice in adrenal medulla, possibly due to impaired adrenocortical corticosterone production.

As expected, we revealed no significant differences in adrenomedullary TH and PNMT immunoprotein levels between CRH (+/+) homozygote and (+/–) heterozygote at the basal conditions. These animals do not suffer from CRH deficiency and their adrenocortical and adrenomedullar functions appear to be physiologically normal.

In summary, we have found difference in adrenomedullary TH and PNMT immunoprotein levels between CRH-KO and CRH (+/+) mice. Physiological relevance of these observations remains to be elucidated.

Acknowledgement

This work was supported by Institute of Experimental Endocrinology Center of Excellence Support ICA1-CT-2000-70008, by grant of the Slovak Grant Agency VEGA 2/2090 and by Fogarty Collaborative Research Award 1R03TW00984.

References

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**, 248-254, 1976

Bruhn TO, Engeland WC, Anthony EL, Gann DS, Jackson IM: Corticotropin-releasing factor in the dog adrenal medulla is secreted in response to hemorrhage. Endocrinol **120**, 25-33, 1987

Hiremagalur B, Nankova B, Nitahara J, Zeman R, Sabban EL: Nicotine increases expression of tyrosine hydroxylase gene. Involvement of protein kinase A-mediated pathway. J Biol Chem **268**, 23074-23711, 1993

Jahng JW, Houpt TA, Joh TH, Wessel TC: Expression of catecholamine-synthesizing enzymes, peptidylglycine alpha-amidating monooxygenase and neuropeptide Y mRNA in the rat adrenal medulla after acute systemic nicotine. J Mol Neurosci **8**, 45-52, 1997

Jeong KH, Jacobson L, Pacak K, Widmaier P, Goldstein DS, Majzoub JA: Impaired basal and restraint-induced epinephrine secretion in corticotropin-releasing hormone deficient mice. Endocrinol **3**, 1142-1150, 2000

Jiang W, Uht R, Bohn MC: Regulation of phenylethanolamine N-methyltransferase (PNMT) mRNA in the rat adrenal medulla by corticosterone. Int J Dev Neurosci **7**, 513-520, 1989

Kilbourne EJ, Nankova BB, Lewis EJ, McMahon A, Osaka H, Sabban DB, Sabban EL: Regulated expression of the tyrosine hydroxylase gene by membrane depolarization. Identification of the responsive element and possible second messengers. J Biol Chem **267**, 7563-7569, 1992

Krizanova O, Micutkova L, Jelokova J, Filipenko M, Sabban EL, Kvetnansky R: Existence of cardiac PNMT mRNA in adult rats: elevation by stress in a glucocorticoid-dependent manner. Am J Physiol Heart Circ Physiol **281**, 1372-1379, 2001

Kvetnansky R, Pacak K, Fukuhara K, Viskupic E, Hiremagalur B, Nankova B, Goldstein DS, Sabban EL, Kopin IJ: Sympathoadrenal system in stress: Interaction with the hypothalamic-pituitary-adrenocortical system. Annals of New York Academy of Sciences **771**, 131-158, 1995

Majzoub JA, Muglia LJ, Jacobson CE, Luedke CE, Karalis K, Pacak K, Jeong K: Regulation of the stress response in corticotropin-releasing hormone deficient mice. In: Stress:Molecular Genetic and Neurobiological Advances (Ed. R. McCarty, G. Aguilera, E. Sabban, R. Kvetňanský), pp.467-470, Gordon and Breach Science, New York 1996

Muglia LJ, Jacobson L, Dikkes P, Majzoub JA: Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature **373**, 427-432, 1995

Muglia LJ, Jacobson L, Luedke CE, Vogt SK, Schaefer ML, Dikkes P, Fukuda S, Sakai Y, Suda T, Majzoub JA: Corticotropin-releasing hormone links pituitary adrenocorticotropin gene expression and release during adrenal insufficiency. J of Clinical Investigation **9**, 1269-1277, 2000

Nagatsu T: Genes for human catecholamine-synthesizing enzymes. Neurosci Res **12**, 315-345, 1991

Sabban EL and Kvetnansky R: Stress-triggered activation of gene expression in catecholaminergic systems: dynamics of transcriptional events. Trends Neurosci. **2**, 91-98, 2001

Thompson RC, Seasholtz AF, Herbert E: Rat corticotropin-releasing hormone gene:sequence and tissue-specific expression. Mol Endocrinol **1**, 363-370, 1987

Udelsman R, Haarwood JP, Millan MA, CHrousos GP, Goldstein DS, Zimlichman R, Catt KJ, Aguilera G: Functional corticotropin releasing factor receptors in the primate peripheral sympathetic nervous system. Nature **319**, 147-150, 1986

Vale W, Rivier C, Brown MR, Spiess J, Koob G, Swanson L, Bilezikjian, Bloom F, Rivier J: Chemical and biological charakterization of corticotropin-releasing factor. Recent Progress in Hormone Research **39**, 245-270, 1983

Vietor I, Rusnak M, Viskupic E, Blazicek P, Sabban EL, Kvetnansky R: Glucoprivation by insulin leads to transsynaptic increase in rat adrenal tyrosine hydroxylase mRNA levels. Eur J Pharmacol **313**, 119-127, 1996

Viskupic E, Kvetnansky R, Sabban EL, Fukuhara K, Weise VK, Kopin IJ, Schwartz JP: Increase in rat adrenal phenylethanolamine N-methyltransferase mRNA level caused by immoblization stress depends on intact pituitary-adrenocortical axis. J Neurochem **63**, 808-814, 1994

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