JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2001, 52, 4, 719—727

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M. SCHWENDT, D. JEZOVA

GENE EXPRESSION OF NMDA RECEPTOR SUBUNITS IN RAT ADRENALS UNDER BASAL AND STRESS CONDITIONS

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovakia

In addition to the central nervous system, glutamate receptors have been recently identified in a number of peripheral tissues, including adrenals. Pharmacological evidence indicates that adrenal glutamate receptors may be involved in stress response, particularly in catecholamine release. However, possible stress-induced changes at the level of local receptors themselves have not been evaluated yet. This study was aimed to investigate gene expression of N-methyl-D-aspartate (NMDA) receptor subunits (NR1, NR2A, NR2B) in rat adrenal gland under basal and stress conditions, using RT-PCR. NR1 mRNA was found to be present in the adrenal gland, while mRNAs coding for NR2-type subunits failed to be detected in adrenal tissue. The distribution of NR1 mRNA in rat adrenals showed higher concentrations in the adrenal medulla (228%) compared to those in the cortex. Single stress stimulus (immobilization) induced a significant increase of NR1 gene expression in both medullar (by 25%) and cortical (by 66%) regions of the adrenal gland at 24 h, while no changes were observed at 3 h after the stress exposure. It is possible that delayed rise in adrenal NR1 gene expression following stress exposure represents one of the factors by which stress exerts long-term effects on adrenal function at the molecular level.

K e y w o r d s : *NMDA receptor, gene expression, mRNA, glutamate, adrenal medulla and cortex, stress.*

INTRODUCTION

Adrenal gland plays a prominent role in the stress response by secreting catecholamines and corticosteroids. Their secretion is regulated by a variety of neurotransmitters and hormones, which may act on local adrenal receptors. Evidence that glutamate may also come into the play originated from the studies reporting the binding of [^{3}H]glutamate to adrenal gland membranes in different animal species, including the rat (1, 2). On the other hand, two studies investigating the presence of genetic message for adrenal glutamate receptors in rats and mice have indicated gene expression of some ionotropic receptor subunits only (3—5). As to the function of glutamate receptors in adrenals, most but not all studies have reported a stimulatory influence of glutamate agonists on catecholamine release *in vivo* and *in vitro* under basal condition (2, 6—8).

In the brain, it is well established that ionotropic glutamate receptors participate in numerous regulatory functions along with central control of hormone release. It has been demonstrated that pharmacological modulations of central glutamate receptors have impact on neuroendocrine response during stress. Pretreatment with centrally acting ionotropic glutamate receptor antagonists inhibited stress-induced responses of several hormones and mediators, such as ACTH, prolactin and catecholamines (9—11). Moreover, it has been found that both acute and repeated stress exposure alter glutamate receptors themselves, as documented by changes in gene expression of ionotropic glutamate receptor subunits in several stress-related regions of the brain, such as hypothalamus, hippocampus and ventral tegmental area (12—14).

Similarly to their function in the brain, adrenal glutamate receptors seem to be involved in the stress response, at least on the basis of pharmacological data. Blockade of N-methyl-D-aspartate (NMDA) receptors by antagonists, which do not cross the blood brain-barrier resulted in an inhibition of stress-induced catecholamine release indicating the involvement of peripheral glutamate receptors (9). In contrast to the brain, no information is available on the effects of stressors at the level of adrenal glutamate receptors themselves.

The present study was aimed to evaluate gene expression of several subunits of NMDA receptor, which has been shown to be important by pharmacological interventions, in rat adrenal gland under basal and stress conditions. A stress paradigm that is known to affect expression of glutamate receptor subunits in the brain was applied.

MATERIALS AND METHODS

Experimental animals and stress procedure

Adult male Sprague-Dawley rats (mean weight 250 g) were maintained under standard conditions with 12 h light-dark circle (light on at 0600) and had water and food available *ad libitum*. One group of animals (n = 6) was exposed to immobilization stress (for 2 hours) by taping all four limbs to metal mounts attached to a solid board. Control group of animals (n = 6) was kept under non-stress conditions. Three hours after the end of immobilization procedure, animals were sacrificed by decapitation. In other series of experiments, animals were killed after a rest for 24 hours. Right adrenal glands were collected and dissected into medullar and cortical part. Tissues were frozen in liquid nitrogen and stored at -80°C until used. All experiments were approved by the Animal Care Committee of the Institute of Experimental Endocrinology.

RNA isolation and RT-PCR analysis

Total RNA from hippocampus and adrenal cortex (medulla) was extracted by the guanidium thiocyanate-phenol/chloroform method (15). Concentration and purity of RNA preparations were measured by absorption spectroscopy. The quality of RNA was judged from the pattern of ribosomal RNA after gel electrophoresis.

Total RNA (1—2 μg) was reverse-transcribed to cDNA using random hexamer primers and avian myeloblastoma virus reverse transcriptase (Promega, USA) as described by the supplier. Aliquots of cDNA (5 μl) were amplified with 2.5 U of *Taq*-DNA polymerase (Promega, USA) in a 25 μl PCR reaction mixture containing: PCR buffer (10 mM Tris-HCl pH 9, 50 mM KCl, 1% Triton-X 100, 3 mM $MgCl_2$), 10 mM dNTP mix and 5—25 pmol of each specific primer. Primers specific for NMDA receptor subunits were designed to amplify with equal efficiency all the described splice variants of the NR1 subunit (bases 461 to 686, clone accession no. L08228), NR2A (bases 600 to 1134, clone accession no. M91561) and NR2B (bases 3306 to 3786, clone accession no. M91562) (*Table 1*). PCR reactions (always performed in the presence of two sets of primers, one for NR1/NR2A/NR2B and other for β-actin) were carried out for 33 cycles, with thermocycle profile: denaturation at 94°C for 30 sec, primer annealing at 55°C for 1 min and primer extension at 72°C for 1 min in the presence or absence of [α-^{32}P]-dCTP (3000 Ci/mmol, ICN, USA). Amplified products were separated on 2% agarose gel, stained with ethidium bromide and photographed under UV illumination with gel-documentation system (Kodak DS 1D). For the measurement of radioactivity

Table 1 Gene-specific primers used for PCR

Type of primer #	5' → 3' sequence	Length (bp)	Amplified fragment (bp)
NR1 FP	CTCATCTCTAGCCAGGTCTA	20	225
NR1 RP	TCGCATCATCTCAAACCAGAC	21	
NMDAR2A FP	ACTCCACACTGCCCATGAAC	20	528
NMDAR2A RP	TTGTTCCCCAAGAGTTTGCTT	21	
NMDAR2B FP	GTTTGATGAAATCGAGCTGGC	21	480
NMDAR2B RP	TCCAGTTCCTGCAGGGAGTT	20	
β-actin FP	TACAACCTCCTTGCAGCTCC	20	280
β-actin RP	ACAATGCCGTGTTCAATGG	19	

FP — front primer; RP — reverse primer;

of ^{32}P-labelled PCR products, separated bands were excised from the agarose gel and allowed to eluate for 2—12 h in a scintillation liquid before counting. A 100 bp marker (Promega, USA) was used as a size standard.

Data analysis

Optical density of PCR products was measured with ”EDAS” densitometric software (Kodak, USA). In each sample, the values for the gene of interest were normalized to housekeeping gene β-actin values, to express relative mRNA levels as arbitrary units. Data were statistically evaluated using two way ANOVA followed by Tukey test to determine differences between groups. Results are presented as means ± S.E.M.

RESULTS

Co-amplification of either of NR1, NR2A or NR2B mRNAs with control template β-actin yielded the products of expected size. While in the hippocampus the genetic message for all NMDA receptor subunits studied was found to be present, only the signal corresponding to NR1 subunit was observed in the adrenal tissue (*Fig. 1*).

Measurements of differences in NR1 mRNA levels among the samples were made using a semiquantitative RT-PCR protocol. In this protocol, the gene of interest (NR1) and the control gene (β-actin) were co-amplified in a single tube. Because correct quantification of PCR products is possible only if both amplified sequences are within the exponential phase of amplification, it was necessary to select an appropriate number of PCR cycles. As shown in ***Fig. 2***, NR1 as well as β-actin fulfilled the mentioned condition in the experimental

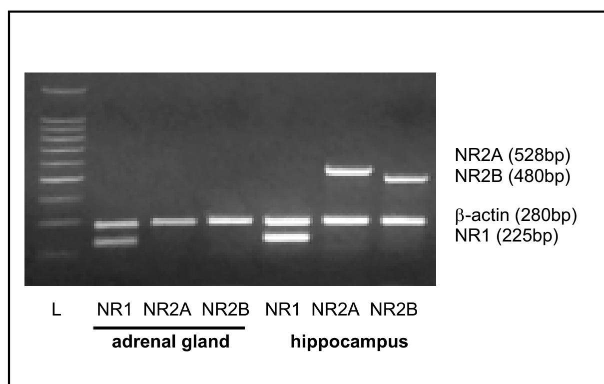

Fig. 1. The profile of expression of selected NMDA receptor subunits in hippocampus and adrenal gland. Analysis of PCR products in 1.8% agarose gel, illustrates that hippocampus express NR1, NR2A and NR2B subunits (as indicated at the bottom and left of the picture), while in adrenals only the signal for NR1 is detected. The control gene β-actin co-amplified with each NR subunit was detected in all samples.

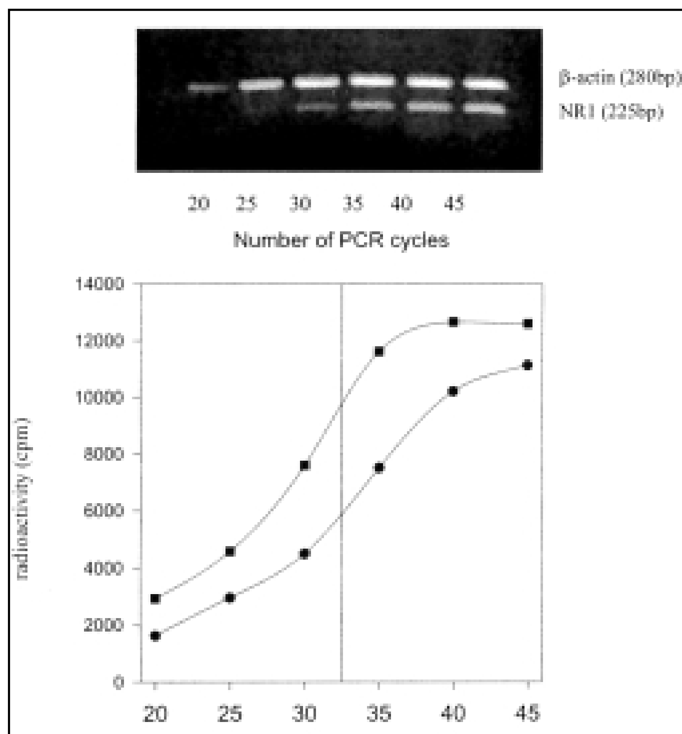

Fig. 2. Quantitative analysis of NR1 with regard to the number of PCR cycles. The top panel illustrates the results of RT-PCR reactions aimed to determine the linearity of NR1 and β-actin cDNA amplification in relation to PCR cycle number. The lower panel depicts the corresponding quantitative data of the ^{32}P-labelled PCR products showed in the top panel. The data expressed in impulses were plotted against the number of cycles to construct the curve illustrating the accumulation of the PCR products.

protocol used. Control RT-PCR reactions, which were performed without RNA template, RT or Taq DNA polymerase remained negative (data not shown).

RT-PCR measurements on separated adrenal medulla and cortex have revealed differential pattern of expression for the NR1 receptor subunit. NR1 mRNA was found to be preferentially located in the medullar region of adrenals (228% comparing to the cortex) (*Fig. 3A*).

The effect of single immobilization stress on the expression of NR1 subunit was measured either 3 or 24 hours following the stress stimulus. After 3 hours, the expression of NR1 failed to manifest any changes (data not shown). Nevertheless, 24 hours after the exposure to single immobilization stress, NR1 mRNA levels were significantly increased in both adrenal cortex (by 66%) and adrenal medulla (by 25%). The mean values of NR1 mRNA levels (normalized to β-actin) obtained in control and stressed groups of rats are presented in *Fig. 3B*. As expected, mRNA levels of the housekeeping gene β-actin were not affected by the stress stimulus (data not shown).

DISCUSSION

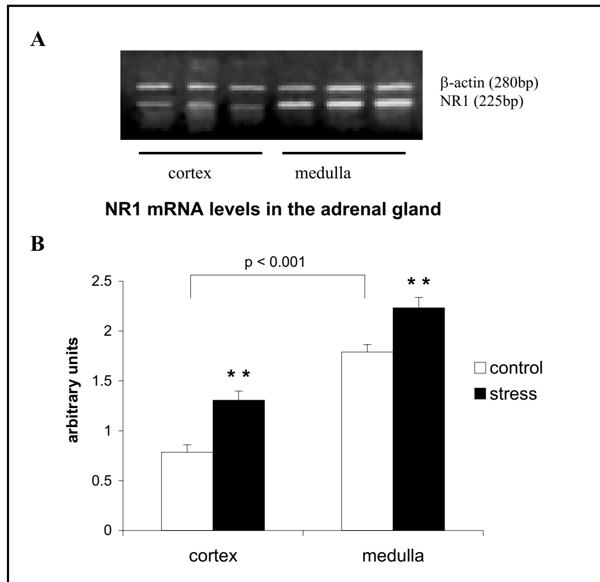

Fig. 3 A — NR1 gene expression in rat adrenal cortex and medulla as detected by RT-PCR in unstressed animals. Agarose gel electrophoresis picture shows signals of co--amplified mRNA for NR1 and control β-actin as indicated on the right side. *B* — The effect of single immobilization stress on NR1 gene expression in rat adrenal cortex and medulla, as measured 24 hours after the stress exposure. mRNA values were normalized to β-actin and expressed as arbitrary units. Values are derived from 6 individual determinations (rats / group) and represent the means ± S.E.M. Statistical analysis was performed by two-way ANOVA with Tukey post-hoc test. ** $P < 0.001$ compared with control values.

The results show that NR1 mRNA, the main NMDA glutamate receptor subunit, is present in the cortex and medulla of rat adrenal gland, while specific mRNAs coding for NR2A and NR2B failed to be detected in adrenal tissue. Under the stress conditions, gene expression of NR1 was found to increase significantly in late time intervals (24 h) after a single stress exposure.

To our knowledge, the presence of NR1 genetic message in rat adrenals has not been documented yet. The obtained data are in agreement with the results of previous studies by Yoneda and Ogita (1, 16) showing characteristics and distribution of glutamate binding sites in rat adrenals. Both the binding studies (1) and NR1 mRNA levels (present experiments) exhibited a similar pattern of signal distribution with a predominant localization in the adrenal medulla. Consistent to these findings in rats is the localization of zeta1 mRNA in the adrenal medulla of mice (4). However, considerable species differences in glutamate binding distribution were reported (2).

Glutamate receptors in the adrenal medulla have been associated with catecholamine release (6—8). *In vitro* experiments on isolated adrenals and cultured chromaffine cells have shown that NMDA have the highest potency amongst glutamate receptor agonists used to stimulate catecholamine secretion under basal conditions (7, 8). The role of NMDA receptors has been supported also by *in vivo* pharmacological studies under both control and stress conditions, showing that manipulation of NMDA receptors is sufficient to inhibit catecholamine response to a stress stimulus (6, 9). Potential significance of NMDA receptors localized in the adrenal cortex is unclear.

Stress-induced rise in NR1 mRNA levels observed in the adrenal gland is similar to the modulation of gene expression of glutamate receptor subunits described previously in the CNS (12). These data together with previous pharmacological studies indicate that not only central but also peripheral glutamate receptors are involved in the stress response. Investigations at two time intervals after the stress exposure revealed that NR1 subunit remained unchanged at 3 h. Significant up-regulation of NR1 mRNA levels in adrenal cortex and medulla was observed 24 hours later, indicating delayed onset of stress-induced alterations. On the other hand, time course of stress-induced activation of gene expression of catecholamine biosynthetic enzymes is known to occur at earlier time intervals after the stress exposure and decreasing back to basal levels about 24 h later (17, 18). It is reasonable to suppose that local glutamate receptors are involved in the control of catecholamine release rather than modulation of local catecholaminergic system via changes in gene expression.

It is generally accepted that NMDA receptors in the CNS are heteromers assembled of subunits from three subfamilies, NR1, NR2A-D and NR3 (19, 20). While the NR1 is expressed ubiquitously in the whole brain, expression of NR2 subunits shows region-specific manner (20). In the present study, genetic message for NR1, but not for NR2A or 2B were detected in rat adrenals. Similar results have already been obtained in the mouse (3). In addition, our preliminary binding experiments with specific NMDA receptor antagonist CGP 39653 (which binds only to heteromeric NR1/NR2A-B NMDA receptors) showed specific binding to membranes isolated from the hippocampus but not from the adrenal gland (Schwendt and Jezova, unpublished results). According to these indications, it is reasonable to suppose formation of unusual homomeric NR1 receptors in rat adrenals. Though function of such homomeric receptors has not been proved except in some *in vitro* studies (21), some authors have suggested distinct properties of peripheral glutamate receptors compared to those located in the brain (22). This suggestion is supported by atypical binding characteristics of glutamate binding sites in adrenals as shown by Yoneda and Ogita (16).

Glutamate acting on local adrenal receptors could origin either from hypothetical glutamatergic innervation of adrenal medulla or from the pool of circulating amino acids (23, 24). Thus, the glutamate, its analogues, food additives and drugs (designed to interact with glutamate receptors in the brain) may exert their action directly on the adrenal gland. The adrenals together with other peripheral organs expressing glutamate receptors could represent potential peripheral targets for neurotransmitter mediated excitotoxicity, food toxicity and should be considered in the therapy (24).

The increase of NR1 gene expression in response to stress could lead to formation of new receptors, which may modulate the sensitivity of adrenal tissue to subsequent stress stimulus or to circulating levels of excitatory amino

acids. Though the molecular mechanisms are not known, observed stress-induced rise in NR1 subunit gene expression seems to represent an important factor by which stress exerts long-term effects on adrenal function.

Acknowledgements: The authors thank Dr. Matute (Universidad del Pais Vasco, Leioa, Spain) for providing the sequences for NR1 and NR2 primers. The study was supported by grants of VEGA 2/6084 and ICA1-CT-2000-70008 of EC.

REFERENCES

1. Yoneda Y, Ogita, K. Localization of glutamate binding sites in rat adrenal medulla. *Brain Res* 1986; 383: 387—391.
2. O'Shea RD, Marley PD, Mercer LD, Beart PM. Biochemical, autoradiographic and functional studies on a unique glutamate binding site in adrenal gland. *J Autonom Nerv Sys* 1992; 40: 71—86.
3. Kristensen P. Different expression of AMPA glutamate receptor mRNA in the rat adrenal gland. *FEBS Lett* 1993; 332: 183—190.
4. Watanabe M, Mishina M, Imoue Y. Distinct gene expression of the N-methyl-D-aspartate receptor channel subunit in peripheral neurons of the mouse sensory ganglia and adrenal gland. *Neurosci Lett* 1994; 165: 183—186.
5. Hinoi E, Ogita K, Takeuchi Y, Ohashi H, Maruyama T, Yoneda Y. Characterization with [^{3}H]quisqualate of group I metabotropic glutamate receptor subtype in rat central and peripheral excitable tissues. *Neurochem Int* 2001; 38: 277—85.
6. Jezova D, Oliver C, Jurčovičová J. Stimulation of adrenocorticotropin but not prolactin and catecholamine release by N-methyl-aspartic acid. *Neuroendocrinology* 1991; 54: 488—492.
7. Nishikawa T, Morita K, Kinjo K, Tsujimoto A. A Stimulation of catecholamine release from isolated adrenal glands by some amino acids. *Jap J Pharmacol* 1982; 32: 291—297.
8. Gonzalez MP, Herrero MT, Vicente S, Oset-Gasque M. Effect of glutamate receptor agonists on catecholamine secretion in bovine chromaffin cells. *Neuroendocrinology* 1998; 67: 181—189.
9. Jezova D, Tokarev D, Rusnák M. Endogenous excitatory amino acids are involved in stress-induced adrenocorticotropin and catecholamine release. *Neuroendocrinology* 1995; 62: 326—332.
10. Zelena D, Makara GB, Jezova D. Simultaneous blockade of two glutamate receptor subtypes (NMDA and AMPA) results in stressor-specific inhibition of prolactin and corticotropin release. *Neuroendocrinology* 1999; 69: 316—323.
11. Tokarev D, Jezova D. Effect of central administration of the non-NMDA receptor antagonist DNQX on ACTH and corticosterone release before and during immobilization stress. *Methods Find Exp Clin Pharmacol* 1997; 19: 323—328.
12. Bartanusz V, Aubry JM, Pagliusi S, Jezova D, Baffi J, Kiss JZ. Stress induced changes in messenger RNA levels of N-methyl-D-aspartate and AMPA receptor subunits in selected regions of the rat hippocampus and hypothalamus. *Neuroscience* 1995; 66: 247—252.
13. Fitzgerald LW, Ortiz J, Hamedani AG, Nestler EJ. Drugs of abuse and stress increase the expression of GluR1 and NR1 glutamate receptor subunits in the rat ventral tegmental area: common adaptations among cross-senziting agents. *J Neurosci* 1996; 16: 274—282.
14. Schwendt M, Jezova D. Gene expression of two glutamate receptor subunits in response to repeated stress exposure in rat hippocampus. *Cell Mol Neurobiol* 2000; 20: 319—329.

15. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156—159.
16. Yoneda, Y, Ogita K. Enhancement of [^{3}H] glutamate binding by N-methyl-D-aspartic acid in rat adrenal. *Brain Res* 1987; 406: 24—31.
17. Nankova B, Kvetňanský R, McMahon A, et al. Induction of tyrosine hydroxylase gene expression by a nonneuronal nonpituitary-mediated mechanism in immobilization stress. *Proc Nat Acad Sci U S A* 1994; 91: 5937—5941.
18. Knopp J, Jezova D, Rusnák M, Jaroščáková I, Farkaš R, Kvetňanský R. Changes in plasma catecholamine and corticosterone levels and gene expression of key enzymes of catecholamine biosynthesis in partially hepatectomized rats. *Endocr Reg* 1999; 33: 145—153.
19. Ishii T, Moriyoshi K, Sugihara H, et al. Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. *J Biol Chem* 1993; 268: 2836—2843.
20. Goebel DJ, Poosch MS. NMDA receptor subunit gene expression in the rat brain: a quantitative analysis of endogenous mRNA levels of NR1Com, NR2A, NR2B, NR2C, NR2D and NR3A. *Brain Res Mol Brain Res* 1999; 69: 164—70.
21. Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S. Molecular cloning and characterization of the rat NMDA receptor. *Nature* 1991; 354: 31—37.
22. Schwendt M, Jezova D. Glutamate receptors and transporters in central and peripheral tissues. *Cesk Fyziol* 2001; 50: 43—56.
23. Llevellyn-Smith TJ, Minson JB, Pilowski PM, Arnolda LF, Chalmers JP. The one hundred percent hypothesis: Glutamate or GABA in synapses on sympatetic preganglion neurons. *Clin Exp Hypertens* 1995; 17: 323—333.
24. Gill SS, Mueller RW, McGuire PF, Pulido OM. Potential target sites in peripheral tissues for excitatory neurotransmission and excitotoxicity. *Toxicol Pathol* 2000; 28: 277—284.

R e c e i v e d: July 23, 2001
A c c e p t e d: October 18, 2001

Author's address: Assoc. Prof. Dr. Daniela Jezova, DSc. Institute of Experimental Endocrinology Slovak Academy of Sciences Vlárska 3, 833 06 Bratislava Slovakia Tel: + 421 7 5477 3800 Fax: + 421 7 5477 4247.

E-mail: ueenjezo@savba.savba.sk