Age related regulation of the hypothalamic pituitary adrenocortical axis in vasopressin deficient Brattleboro rats

Doctoral theses

János Varga

Semmelweis University
János Szentágothai PhD School of Neurosciencies

Supervisor: Dr. Dóra Zelena scientific supervisor, Ph.D.

Official referees: Dr. István Gyertyán research group leader, Ph.D.
Dr. István Gacsályi senior research fellow, Ph.D.

Chairman of committee of comps: Dr. Erzsébet Ligeti professor emeritus, member of the Hungarian Academy of Sciences
Members of committee of comps: Dr. Tibor Bartha professor, D.Sc.
Dr. Gergely Zachar scientific supervisor, Ph.D.

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Introduction

The constant chronic stress weakens the immune system and may contribute to a number of physical diseases (eg, stomach ulcers, diabetes, and cancer) as well as to development of mental illnesses (depression, anxiety).

Scientists have long been concerned about that maternal stress can affect pregnancy and subsequent infant development. The investigation of fetal and perinatal stress physiological processes is necessary for a better understanding of the issues, especially the functioning of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis reaches its adult form through maturation processes, significant functional changes take place during development, and its sensitivity is heavily influenced by fetal, perinatal life events. The increased fetal stress axis activity can cause lower birth weight and attention disorders. During the perinatal period it was wildly observed that different stressors applied in infants lead only moderate HPA axis stimulation compared to adults, therefore this age is called stress hyporesponsive period (SHRP). Although the underlining mechanisms are unclear, decreased production and transport of hypothalamic hormones may contribute to its development.

Perinatally the corticotropin releasing hormone (CRH) gen expression is smaller compared to adults, in contrast with vasopressin (AVP) expression, which reaches its mature form very early during the development. Probably AVP can be a key regulator of the HPA axis during the SHRP. The aim of my work was to examine the age-related role of AVP in the stress processes.

Aims

1. The role of AVP in the regulation the HPA axis during long-term **chronic mild stress** (CMS 5 weeks) in adult Brattleboro rats.
2. The **age-related** role of AVP in the regulation of HPA axis by direct comparison of adult and perinatal rats
    a. in Brattleboro rats (hypoglycaemia, lipopolysaccharide (LPS) treatment).
    b. by V1b antagonist and AVP antiserum pretreatment (LPS).
    c. age related CRH sensitivity of pituitary.

3. ACTH - glucocorticoid dissociation

Possible explanations:

    a. Different timing of adrenocorticotropin (ACTH) and corticosterone secretion (10 min maternal separation).
    b. Transcortin level differencies.
    c. Different ACTH sensitivity of the adrenal glands.

Possible mechanisms:

    d. ACTH independent corticosterone regulation, role of catecholamines, especially β-adrenoceptors.
    e. Age-related comparison of corticosterone and aldosterone levels in AVP-deficient and control animals (hypoglycaemia).

4. Since the **age-related** differences were AVP independent **gluco-and mineralocorticoid secretion** was studied in Wistar rats, too.
    a. Hormone levels measurements during stress processes (LPS, hypoglycemia).
    b. Age-related receptor and enzyme differences (PCR, immunocytochemistry).

Methods

Animals

Brattleboro rats (male adults: 7–12 weeks old; pups: 7-8 or 10-day-old) were maintained at the Institute of Experimental Medicine in a colony started from breeder rats (Harlan, Indianapolis, USA). We compared the AVP-deficient homozygous (AVP-) rats with congenital diabetes insipidus to heterozygous (AVP+) control rats from the same litters. We also used male Wistar rats to confirm the age related differences. All studies were carried out in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) and were approved by the Animal Welfare Committee of the Institute of Experimental Medicine, Budapest, Hungary.

1. Chronic unpredictable stress (CMS)

CMS consisted of different mild stimuli for 5 weeks. The procedures were conducted twice a day, from 9h and from 16h (holeboard, increased number of animals per cage, 1h restraint, wire suspension, rotarod, 18h water deprivation, 24h food deprivation, 30min restraint on ice, stationary beam, wet bedding (100ml water in cage), 45min restraint, elevated plus maze for 5min, 15-5min forced swim test). The animals were decapitated at least 24 after the last stimulus during resting conditions. Blood samples were collected and ACTH, corticosterone levels were measured by radioimmunoassay (RIA).

2. Age related stress axis regulation in Brattleboro rats

Lipopolysaccharide (LPS, bacterial infection model) treatment

Groups of adults or 10-day-old pups were injected intraperitoneally (ip) with LPS (100mg/l ml/kg in saline, SIGMA, O55:B5) or 0.9% saline. The pups were marked with

ink and returned to their mothers. Two hours later the rats were decapitated and trunk blood was collected on ice for hormone measurement (ACTH, corticosterone) and the pituitaries of pups were collected in 100µl 0.1N HCl for assessment of the genotype by AVP measurements.

To confirm the AVP related differencies, groups of normal AVP producing adult or 10-day-old Brattleboro rats were injected ip with a V1b receptor antagonist (SSR149415; 10 mg/1 ml/kg) or vehicle (0.9% saline with a few drops of Tween 80) 15 min before LPS injection.

In a separate experiment AVP antiserum (20µl from a 40 mg/ml solution) or normal rabbit serum (NRS) was also injected ip followed 15 min later by saline or LPS treatment in normal AVP producing Brattleboro pups.

Stress axis activation by insulin induced hypoglycemia

Stress of hypoglycemia was induced by ip insulin injection (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) at the dose of 3NE/1ml/kg in saline in fasting rats. Adult rats were fasted for 18h while 10-day-old pups for only 4h to achieve comparable degree of starvation. Rats of control groups were injected with the same volume of vehicle (0.9% NaCl). Animals were decapitated 90min later. Blood glucose levels were measured by commercially available analyser (D-Cont Personal, 77 Elektronika Kft., Budapest, Hungary) at the time of decapitation. Trunk blood was collected for ACTH and corticosterone measurements.

CRH sensitivity of hypophysis in vitro static incubation

Each gland was chopped into small pieces with a sterile scalpel blade and put into 1 ml DMEM. After changing the medium, the pieces were preincubated for 1 h at 37°C under a 95% O_2–5% CO_2 atmosphere when DMEM was replaced by a fresh solution for another 1h. After preincubation, the medium was changed above the fragments and collected every 15 min

for 6 times, adding 10^{-10}M CRH to the second fraction. At the end of the experiment media were removed, centrifuged at 3000g for 5 min and the supernatant was stored at -20°C until ACTH measurement.

3. ACTH independent glucocorticoid secretion

Timing of sample collection – 10 minutes maternal separation

Samples were collected after 10 minutes maternal separation of 7-8-day-old AVP deficient, or normal AVP producing pups from the same litter. The genotypes of the pups were determined by their hypophysis AVP content measured by RIA. Plasma ACTH and corticosterone were measured at the end of experiment.

Corticosterone binding capacity

Transcortin (CBG) binding capacity was determined using the modified Sephadex-LH-20 based method.

ACTH sensitivity of the adrenal cortex

The in vitro incubation protocol was described at the CRH sensitivity. This time 10^{-12}M ACTH was added during the second 15 minutes period to each separate adrenal gland and corticosterone was determined from the supernatant.

Role of catecholamines

β adrenerg antagonist: in vivo measurements

The 10-day-old di/di Brattleboro rat pup was used as a model organism where corticosterone secretion to stressors occured without previous ACTH elevation. Here we used the Actrapid-induced hypoglycemia model. After 4h maternal separation saline or 2.5 mg/ml/kg propranolol (β-receptor antagonist) was administrated ip. 15 min later a further ip injection was introduced either with saline or with Actrapid

(3NE/ml/kg, a rapid insulin). 90 min later the pups were decapitated and trunk blood was collected for corticosterone measured by RIA, while blood glucose was measured immediately with a commercially available analyzer.

β adrenerg antagonist: in vitro measurements

We used the incubation protocol described at CRH sensitivity. Control medium, 10^{-10}M ACTH, 10^{-5}M Propranolol, or ACTH + Propranolol was added during the second 15 minutes period. Corticosterone was determined from the supernatant.

Gluco- and mineralocorticoid levels in Brattleboro rats

Aldosterone levels were also measured during „Stress axis activation by insulin induced hypoglycemia ” experiment.

4. Age related alterations of gluco- and mineralocorticoid levels in Wistar rats

LPS treatment

The same protocol was used in Wistar rats as previously described for Brattleboro rats. Plasma ACTH, corticosterone, renin and aldosterone levels were measured by RIA.

Stress axis activation by insulin induced hypoglycemia

The same protocol was used in Wistar rats as previously described in Brattleboro rats. Plasma ACTH, corticosterone, renin and aldosterone levels were measured by RIA.

Real-time PCR

Total RNA was isolated from homogenates of the hypothalamus and hippocampus using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and then converted to cDNA by High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA). The cDNA samples

were pooled for each group and measured in duplicates. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Life Technologies) on ABI StepOnePlus instrument according to the manufacturer's instructions. Primers used for the comparative CT experiments were designed by the Primer Express 3.0 program. Melting curve analysis to confirm the identity of PCR products has been performed by using ABI StepOnePlus instrument's Software v.2.1 according to the instructions of the manufacturer. Gene expression was analyzed by ABI StepOne Software 2.1 program. GAPDH was used as endogenous control. Relative quantity of mRNAs was referred to corresponding samples of the adult Wistar rats based upon the $2^{-\Delta\Delta CT}$ method.

Sample collection

After decapitation, trunk blood was collected on ice-cold tubes and after centrifugation (3000 rpm/min 20 minutes) the serum was stored at -20°C.

Tissue samples of macrodissected brain regions were collected under RNase free conditions from unstressed adult and postnatal rats and were kept at -70°C until PCR measurements.

Hormone measurements

Plasma ACTH concentrations were measured by RIA in 50µl unextracted plasma. ACTH antibody (no. 8514) was developed against h-$ACTH_{1–39}$. Concentrations of plasma corticosterone were measured in 10 µl unextracted plasma by RIA. Corticosterone antibody was developed against corticosterone-3-carboxymethyloxime-bovine serum albumin. Plasma aldosterone levels and plasma renin activity were measured using RIA Aldosterone kit and Angiotensin I RIA kit (Immunotech, France) The same RIAs were used to measure corticosterone and aldosterone concentrations in the incubation medias.

Statistical analysis

Data were analyzed by three-way ANOVA for factor age, and by two-way ANOVA for factor genotype / treatment and stressor, or in the case of in vitro experiments by repeated measure ANOVA using the Statistica 8.0 program of StatSoft, Inc., Tulsa, OK, USA.Post hoc comparison of the data from different experimental groups was performed by the New-man – Keuls test.

Results

1. Role of AVP during chronic unpredictable stress

The genotype had no general effect on plasma corticosterone levels. In contrast, CMS significantly elevated their levels. However, the effect of CMS was different in the two genotypes, in AVP+ rats the corticosterone level was significantly elevated, but in AVP- rats it remained stable. Our CMS observations supports the enhanced regulatory role of AVP during chronic stress.

2. Age related regulatory role of AVP during acute stress

In the adults, serum corticosterone concentrations increased markedly after LPS and Actrapid injection and there was no significant difference between the AVP+ and AVP- rats. Similarly, serum ACTH concentrations increased markedly after LPS injection without genotype effect or interaction. On the other hand, after Actrapid treatment AVP- adult rats showed only 50% of the ACTH elevation of AVP+ rats.

In 10-day-old AVP+ pups the serum ACTH concentrations showed significant elevations to LPS and Actrapid injections. On the contrary, in the AVP- pups stressors failed to induce significant elevation in the circulating ACTH concentrations. In pups, the serum corticosterone concentrations showed significant effects both for stressor treatment and genotype. The corticosterone concentrations showed greater elevation in the AVP- rats. We confirmed these observations with V1b antagonist, and AVP antiserum pretreatment, too.

Since the ACTH increase of 10-day-old pups (AVP+) was similar to the elevation observed in adults, but the corticosterone elevations was significantly smaller, we can

conclude that the reduced stress axis reactivity during SHRP affects mostly the corticosterone.

The in vitro ACTH release was much higher in adults than in the 10-day-old Brattleboro rat pups, the same CRH concentration resulted in 600-800% increase in adults and only in 50% increase in pups. Thus, an other hypothalamic factor (AVP) may be responsible for the ACTH response during perinatal period.

3. ACTH independent glucocorticoid secretion

Although the AVP can be the main regulator of ACTH secretion during the perinatal period, it does not have the same effect on corticosterone levels. Dissociation between ACTH and glucocorticoid levels was observable during our experiments.

Possible explanations

3/1 Timing of sample collection

An important issue is the timing of sampling. During stress processes the corticosterone response followes the ACTH response with a delay, the amplitude of their production slips in time from each other, so the wrong timing of sample collection can lead to contradictory results. We observed similar dissociation during 3 different experiment (sample collection 10 min, 90 min and 120 min after treatment), so the dissociation can not be explained with the different timing of sample collection.

3/2 Corticosterone binding capacity

By RIA total corticosterone levels are measured instead of free levels. If the transcortin levels differ according to age and genotype, the free corticosterone levels may correlate better with ACTH levels than total corticosterone levels. CBG

in the serum of 10-day-old pups was significantly greater (20%) in the absence of AVP. The adult rats had higher (30%) CBG, but in adulthood there was no difference between AVP+ and AVP- rats. The enhanced CBG capacity of AVP- pups can contribute to their higher total corticosterone concentrations, but is unable to explain the stressor-induced elevations and the differences in adults.

3/3. ACTH sensitivity of the adrenal cortex

Baseline in vitro corticosterone secretion was significantly greater from adult adrenals than from pup adrenals, reflecting the differences in adrenal gland weight. In adults neither the genotype nor the treatment - genotype interaction was significant suggesting a similar corticosterone secretion capability of AVP+ and AVP- adult rat adrenals. The adrenal glands of AVP- pups showed greater ACTH sensitivity, but the difference is small (25-30%) to fully explain the observed ACTH – corticosterone dissotiation.

Possible mechanisms

The AVP- pups showed huge (third of the increase observed in adults) corticosterone elevation without high ACTH increase. Since neither the transcortin level differences, nor the altered ACTH sensitivity of adrenal glands did fully explain the observed ACTH – corticosterone dissociation, it raises the possibility of ACTH-independent corticosterone regulation.

3/4 ACTH independent glucocorticoid secretion - catecholamines

We have shown with the hypoglycemic stressor that propranolol (2.5 mg/kg; a beta-receptor antagonist) significantly reduced the stressor-induced corticosterone

elevation in AVP- rats. Our in vitro experiments confirmed that this effect is direct on adrenal gland. So sympathetic adrenomedullary system seems to be a key element of corticosterone release during stress processes.

3/5 Gluco- and mineralocorticoid levels in Brattleboro rats

Glucocorticoids are structurally very similar to mineralocorticoids and they can act on the same receptors. There is no doubt that ACTH can also trigger aldosterone release during acute and chronic stress. An alternative hypothesises of the ACTH – corticosterone dissociation among genotypes is that in plasma the gluco- and mineralocorticoid levels together follows the ACTH level changes. However we did not find genotype related differencies in aldosterone production, but we detected higher aldosterone increase in perinatal than in agult age. We can conclude that during SHRP only the glucocorticoid secretion is damped and mineralocorticoids more effectively react to stress. We confirmed our idea also in Wistar rats.

4. Age related alterations of gluco- and mineralocorticoid levels in Wistar rats

In 10-day-old Wistar rat pups, which exhibited the well known reduction of stress-induced corticosterone release during SHRP, stress-induced elevation in aldosterone concentrations were significantly higher compared to those in adulthood. The stressor-induced aldosterone increase during the perinatal period suggest a shift in the balance between stress-induced glucocorticoid and mineralocorticoid hormone release during the development.

Potential functional significance of enhanced aldosterone activity during postnatal period is supported by concomitant higher 11β-hydroxysteroid dehydrogenase

(HSD)2 (glucocorticoid deactivating enzyme) and lower 11β-HSD1 (glucocorticoid activating enzyme) mRNA levels in the observed brain areas of pups compared to adults. Moreoever, both the gluco- and mineralocorticoid levels were low in pups keeping the glucocorticoid effect of its nadir.

Conclusions

1. According to some theory during chronic stress AVP becomes the main regulator of the stress axis. Our previous work did not confirm this using two weeks stressors, but during prolonged stimulation (e.g. 5 weeks chronic mild stress) the regulatory role of AVP came into highlight.
2. We established that during the perinatal period AVP is the main regulator of the ACTH secretion with direct comparison of adult and 10-day-old rats control and AVP-deficient Brattleboro using two different stressor (LPS and Actrapid-induced hypoglycaemia). The results were confirmed with pharmacological (V1b antagonist SSR149415) and immunological (AVP antiserum) blockade. The hypophysis of 10-day-old animals were less sensitive to CRH stimulation emphasizing the regulatory role of AVP.
3. In most cases the reduced ACTH secretion in AVP-deficient rats was not accompanied by smaller corticosterone secretion. (A) First we wanted to close out technical problems. (1) Already during 10 min maternal separation the reduced ACTH secretion of AVP-deficient rat pups were accompanied by higher corticosterone leves. This suggest that the different timecurve of the two hormones cannot be responsible for the discrepancies. (2) The enhanced corticosterone binding globuline levels are also not responsible for the ACTH-corticosterone discrepancies in adult AVP-deficient rats and only partly explain it in pups. (3) Similarly, changes in adrenal gland ACTH sensitivity can explain only part of the discrepancies in pups but not in adults. (B) As we established that there is an ACTH-independent glucocorticoid secretion we examined some possible mechanisms. (1) We confirmed both in vivo and in vitro that catecholamine regulates glucocorticoid secretion at the level of the adrenal gland. (2) Although our hypothesis that gluco- and mineralocorticoid together follow

the ACTH secretion failed, but a surprising result suggested a major role of mineralocorticoid in perinatal stress reaction.
4. In an other rat strain (Wistar) with two different stressor (LPS, hypoglycaemia) we confirmed that during the perinatal period aldosterone is the main stresshormone. During this period the body keeps the effect of glucocorticoids on a minimum level by low GR and MR receptor and 11-β-HSD1 and high 11-β-HSD2 (degrading enzyme) level.

Publications

1. Publications related to the theses

Varga J, Domokos A, Barna I, Jankord R, Bagdy G, Zelena D. (2011) Lack of vasopressin does not prevent the behavioural and endocrine changes induced by chronic unpredictable stress. Brain Res Bull. 84(1):45-52.

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2. Other publications

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