The role of vasopressin on regulation of acute and chronic stress in Brattleboro rats

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PhD Thesis

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Introduction

The World Health Organization predicts that within a decade the stress-related psychological disorders (especially depression) will be the most common diseases in the developed world. These assumptions emphasize the importance of stress studies.

Stress is defined by life sciences as a specific disease state. Hans Selye formulated the "general adaptation syndrome" theory and concept of "stress" entering into the history of lifescience forever. Selye, while trying to identify a specific hormonal effect of different stimuli, observed that even though we have 'attacked' the organism with a specific stimulus the response will be general and the same for all kind of stimulus. This response cannot be maintained for a long time and if the stimulus persists, resistance will be developed, while the body tries to adapt to the changing circumstances. Selye called the provoking factors as stressors and the condition was named stress. Although various stressors activate different brain areas in the central nervous system, the final common pathway is in the hypothalamus, the parvocellular corticotropin-releasing hormone (CRH) producing neurons. It is important to note that another neuropeptide, vasopressin (AVP) can be detected not only in the magnocellular, but also in the parvocellular neurons. In rats almost half of parvocellular neurons coexpresses AVP and CRH. AVP is a weak ACTH secretagogue, but it can enhance the ACTH-releasing effect of CRH. This was demonstrated by in vivo and in vitro experiments. After acute and chronic stress the CRH/AVP ratio might change. Some authors believe that the function of elevated AVP level is to maintain the hypothalamo-pituitary-adrenocortical-axis (HPA, stress-axis) functionality among chronic stress. Although many studies dealt with the effect of AVP on HPA axis, but its physiological role has not yet developed clearly. We may get more detailed picture about the role of AVP in HPA axis regulation if we investigate the effect of various (intensity and modality) stressors in the same animal model. For that purpose the Brattleboro strain seems to be the most suitable one. This strain was discovered in 1962 in the town of Brattleboro as a consequence of a natural point mutation in the Long Evans strain. Because the lack of AVP is its inborn quality we do not need any extra treatment to get rid of systemic effect of AVP. It means that animals do not have to suffer from additional stressful stimuli (injection of an antagonist, antiserum, lesion in brain etc.). Examination of these animals might bring us closer to understanding the role of AVP in regulation of acute and chronic stress.

Aims

A. To investigate the role of AVP in acute stress

1. *examination of stress response of AVP-deficient rats to different acute stressful stimuli*
2. examination of stress response during 10 minutes forces swim test in AVP-deficient Brattleboro rats
3. AVP deficiency and acute opiate effects on HPA axis activity in Brattleboro rats

B. To investigate role of AVP in chronic stress

1. *the effect of AVP on the development of chronic stress state after chronic morphine administration*
2. the effect of AVP on the development of HPA axis changes during 28 days restraint stress
3. investigation of the effect of AVP on chronic mild stress- induced HPA axis and behavioral changes

C. To investigate role of AVP during the perinatal period

1. *investigation of stress reactivity in Brattleboro pups*
2. investigation of stress reactivity of male and female rat pups

Material and methods

Brattleboro rats

We compared homozygous (di/di) AVP-deficient rats with congenital diabetes insipidus with heterozygous (di/+) control rats from the same litters. Central diabetes insipidus is a result of single nucleotide mutation in the preproAVP gene. Rats were maintained in a controlled environment (temperature23± 1°C, humidity 50–70%, day/night schedule of 12/12 h with lights on at 07.00 h) and were fed with commercial rat chow (Charles River, Budapest, Hungary) and had free access to tapwater. Due to the extremely high urine production in di/di rats, their sawdust bedding was changed daily.

Sample collections

- Vena jugularis canullation

Two days before our experiment the vena jugularis dexter was exposed by blunt dissection and a catheter consisting of PE-50 tubing with a tip of silicone tubing (medical-grade silicone tubing; ID 0,64mm; OD 1,2 mm Dow Corning; MI; USA) was inserted in cordal direction and ligated to the vessel. The tubing was exteriorized in the neck and filled with heparinized saline solution.

- Blood and organ samples

After decapitation brains were removed and were frozen immediately on dry-ice, pituitary glands were removed and replaced in freezing medium on dry-ice, too. Brain and pituitary samples were stored at -70°C. Trunk blood (7-10 ml) was collected into ice-cold K_2-EDTA-containing (150µl 20 w/v % EDTA) tubes and after centrifugation the plasma was stored at -20°C. Thymus and adrenal glands were collected into preweighted plastic tubes.

- Hormone measurements

Plasma ACTH was measured by radioimmunoassay (RIA) from 50µl unextracted plasma using a specific antiserum developed at the Institute of Experimental Medicine. Plasma corticosterone was measured from 10µl unextracted plasma by RIA using a specific antiserum developed at the Institute of Experimental Medicine. All samples from a single experiment were measured in the same RIA.

- *In situ* hybridization

Frozen forebrain and pituitary tissues were mounted on a cryostat microtome and cut into 16µm coronal sections. Every sixth brain section was mounted on a silanized slide, from anterior commissure to the end of the amygdala. Six pituitary sections were put on one slide. CRH and AVP mRNA levels were quantified by [35S]UTP-containing riboprobes complementary to exonic sequences of the genes (the plasmids containing 1.2 kb template was a generous gift of Dr K Majo, Northwestern University). POMC mRNA levels were quantified by riboprobes complementary to the exonic sequences of the POMC gene in the presence of [35S]UTP (the plasmid containing 1.2 kb template was a generous gift from Dr J Eberwine, University of Pennsylvania). The radioactive signal was detected by an image plate with the help of a fluorescent picture analyser (FLA 000, Fujifilm, resolution 50 µm). After digitalization the intensity and area of the signal was calculated by the ImageJ software.

- Histological examination of the adrenal cortex

15µm sections of paraffin-embedded adrenal glands were mounted on gelatin coated slides and were stained by hematoxylin-eosin. The depth of the adrenal cortex was calculated by using the Image J analyzer. We subtracted the area of the medulla from the area of the whole adrenal gland at its highest diameter.

- Static incubation

Adrenal glands were obtained after decapitation. Each gland was chopped into eight pieces and preincubated in 1 ml DMEM (Sigma–Aldrich) containing 2.5 g BSA/l at 37 °C under 95% O2– 5% CO2 atmosphere for 2x1 h. Than the medium was collected and replaced with fresh media every 15 min six times with different doses of ACTH (10^{-11} M; 10^{-12} M; 10^{-13} M) added to DMEM of the second fraction.

Experiments

Acute stress studies

- Novelty stress

Rats were placed into an open plastic cage (40x36x20 cm) without bedding material for 10 min. This cage is bigger than the home-cage (30*36*20 cm) and was previously defecated by another rat to induce more severe anxiety. Non-handled controls and stressed animals were decapitated at the end of 10 min novelty exposure.

- Restraint stress

The animals were cannulated as described. Two days later a pre-stress blood sample was collected (0 min) through the jugular catether without touching the animal then the animals were subjected to restraint stress in a polyethylene tube. Stressor duration was 1 h and blood samples were collected before (at 0 min), during (at 5, 15, 30, 45, 60) and after (at 120 min) restraint stress.

Chronic stress

Adult male rats got morphine injection twice a day for 16 days. The injections were stated with 10mg/kg than during the first 10 days the dose was increased by 10mg/kg per day and the 100mg/kg dose was maintained for

the last 6 days. The controls received 0.9% saline (0.2 ml/100 g sc). On the morning of decapitation (at the time of last injection), we took blood from the tail under slight restraint for 2 min, cutting it with a sharp surgical knife. Half of previously morphine-treated animals got only saline (MS group, longer withdrawal, 16 h), while the other half were treated with 100 mg/kg morphine (MM group, 4 h withdrawal) and the animals were decapitated 4h later

Perinatal period

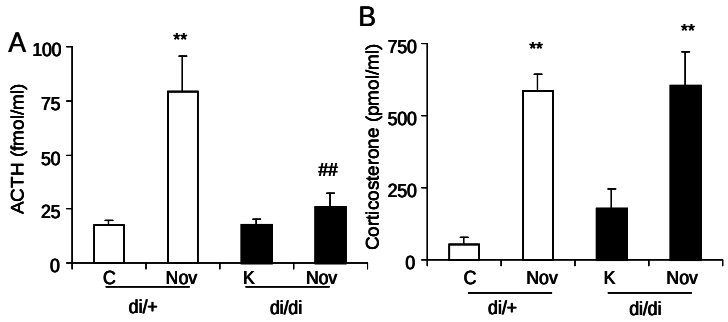
We investigated the effect of 1, 4, 12 and 24-h long maternal deprivation on brain CRH mRNA, as well as on plasma ACTH and corticosterone levels in 9-d-old pups. In each litter, half of the pups were separated from their mother, whereas the other half remained undisturbed. During maternal deprivation, littermates were kept together in a novel cage with normal bedding. No additional treatment was provided. All pups were killed by decapitation.

Results

Acute stress studies

Novelty stress

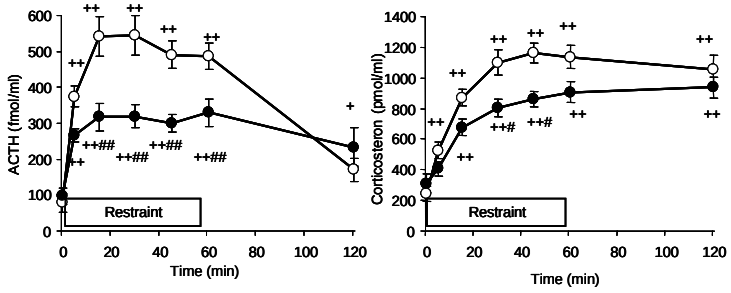
In di/+ animals, both the ACTH and corticosterone plasma levels were elevated at the end of 10 min novelty stress. The resting plasma levels were similar in di/+ and di/di animals. Novelty stress did not induce ACTH elevations in di/di rats, however, the rise in corticosterone was similar to that seen in di/+ animals.



Novelty stress A: ACTH levels , B: Corticostrone levels
C-control, unstressed; Nov- at the end of 10 min novelty
**** p< 0,01** versus control; **## p<0,01** versus di/+.

Restraint stress

Stress stimulated both ACTH and corticosterone secretion within 5 min. The highest ACTH levels were visible at 15 min and remained stable until the end of the stress (1 h) but went back to basal levels by 120 min. By contrast, the corticosterone levels elevated gradually throughout the stimulus and remained stable even 1 h after stressor cessation. The AVP-deficient rats revealed smaller elevations during the stress for both hormone.



Serial blood sampling from cannulated Brattleboro rats during **1 h restraint**

+p<0.01 vs 0 min + +p<0.05;; #P<0.05, ## p<0.01 versus di/C.

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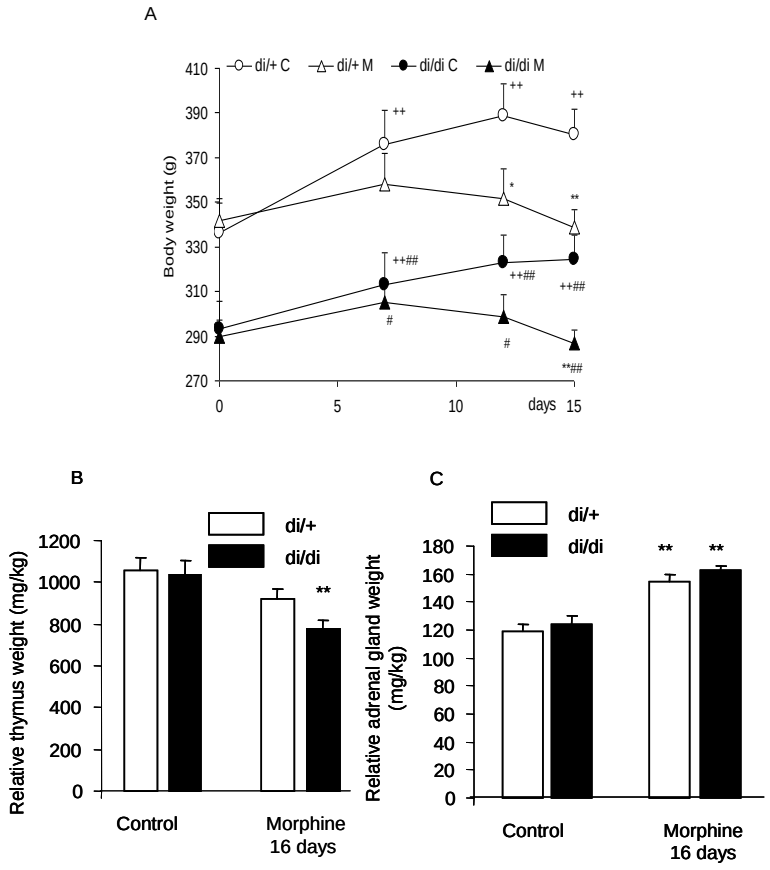
Chronic stress studies

Somatic changes

The saline-treated animals of both genotypes gained weight normally while the repeated morphine treatment induced weight reduction. There was no interaction between the treatment and the genotype suggesting that the body weight reduction was present both in di/+ and di/di rats to a similar extent. As a sign of chronic stress, repeated morphine injections induced thymus involution without genotype effect. By pairwise comparison the AVP deficiency aggravated the reduction of the thymus weight, so surely not prevented the development of this symptom. Due to enhanced glucocorticoid synthesis adrenal gland hyperplasia was also visible in repeatedly morphine-treated without any effect of genotype (A, B, and C pictures).

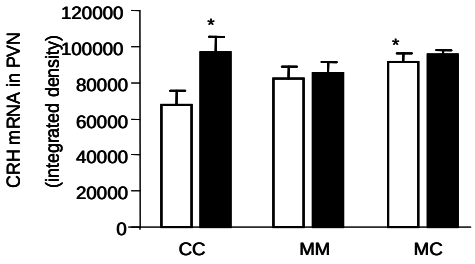


Effect of chronic morhine treatment on somatic parameters
++p<0,01 versus initial weight; *p<0,05 **p<0,01 versus saline treated;
#p<0,05 ##p<0,01 versus respective di/C group
A=Body weight, B= relative thymus weight, C=relative adrenal gland weight

Changes in HPA-axis

CRH mRNA level

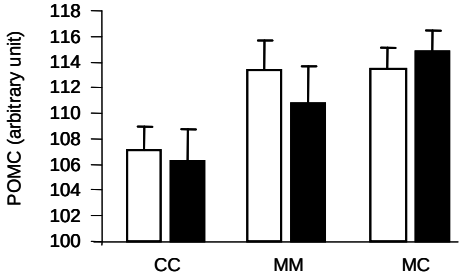
The intensity of the CRH mRNA signal above the whole nucleus paraventricularis hypothalami (PVN) was gradually increased in repeatedly morphine injected groups with a significant rise 16 h after the last treatment. Higher elevation was induced by the AVP deficiency itself. In this genotype the morphine treatment did not induce further changes.



CRH mRNA level in the PVN
CC, saline treatment; MM, morphine treatment, decapitation 4 h after the last injection; MC, repeated morphine treatment, only the last injection
was saline, i.e., decapitated 16 h after the last morphine injection. *P<0.05 versus saline treated

Changes of POMC mRNA levels in the anterior lobe of the pituitary

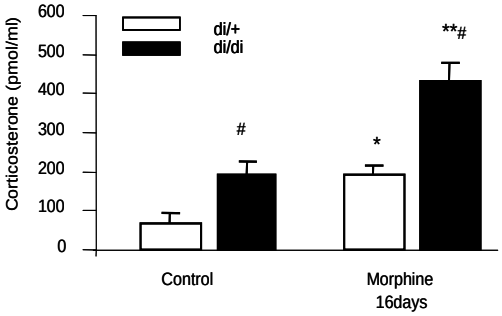
Repeated morphine injection significantly elevated the POMC mRNA level in the anterior lobe of the pituitary measurable at both 4 h (MM group) and 16 h (MC group) after the last morphine injection (main effect of treatment p<0,01). .Both the basal and stressed levels were the same in control and AVP-deficient animals.



POMC mRNA levels **in the anterior lobe of the pituitary**

Resting corticosterone levels

The resting corticosterone level of di/+ rats was elevated by the time, when the last injection was expected. Although the saline-treated di/di group had already higher resting levels, but repeated morphine treatment was able to induce a further rise.

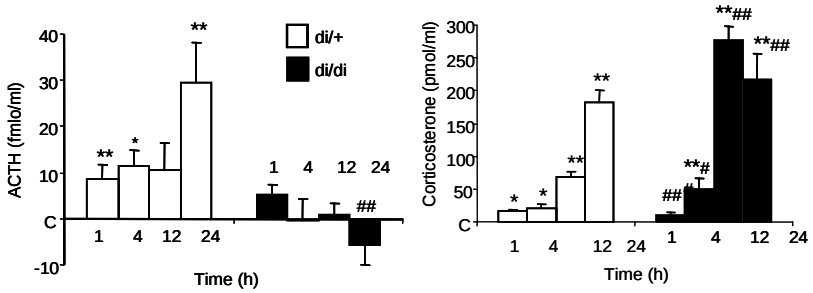


Plasma corticosterone levels in the tail blood at the time of the last injection was expected
*p<0,05 **p<0,01 versus saline treated;
#p<0,05 versus di/+ group

Effect of AVP during the perinatal period

The time course of the effects of maternal separation in 9-d-old rats

In control di/+ rat pups, plasma ACTH levels showed a small but significant elevation as a result of separation at all studied timepoint, the highest level being seen after 24 h. In undisturbed pups, there was no difference in plasma ACTH levels between the genotypes. In di/di rats, the ACTH levels did not change after separation. The separation induced a significant stepwise corticosterone elevation. The undisturbed di/di offspring had higher corticosterone plasma levels during the whole examination period. The maternal separation-induced corticosterone elevation was significantly larger in di/di than in di/+ littermates, starting from 4 h after separation, suggesting that the effect of the AVP deficiency was synergistic with the separation-induced corticosterone increase.

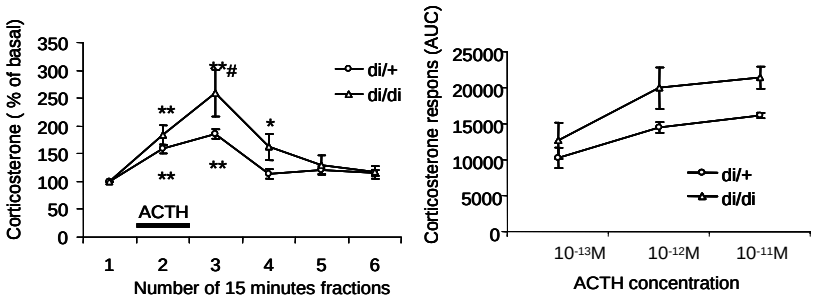


The time course of the effects of maternal separation in 9-d-old rats

*p<0,05 **p<0,01 versus control pups, ##p<0,01 versus di/+ pups

In vitro ACTH-induced corticosterone responses in 10-d-old pups

Three doses of ACTH (10^{-13}M, 10^{-12}M, and 10^{-11}M) dose dependently increased the area under the curve of corticosterone secretion. The increase was higher in di/di. Yet, the difference was not significant at any time point in *post hoc* comparisons. The lowest dose, which most likely reveals the enhancement in sensitivity, significantly elevated corticosterone secretion from the adrenal gland. The increase was significantly larger in di/di pups 15 min after ACTH treatment, but not at other time.



The in vitro ACTH sensitivity of the adrenal gland

*p<0,05 **p<0,01 versus control pups, #p<0,05 versus di/+ pups

Discussion

Acute stress

AVP has an important, but stressor-specific role in regulation of ACTH secretion. We observed three types of HPA responses in AVP-deficient rats: 1) stressors with decreased ACTH and corticosterone response (for e.g. morphine injection, aggression, restraint), 2) stressors with decreased ACTH response but no change in corticosterone (for e.g. novelty, EPM, forced swim, hypoglycaemia, egg white), 3) stimuli without a change in ACTH or corticosterone (for e.g. social avoidance, footshock, ether inhalation). It has been hypothesized that the brain categorizes stressors and utilizes neural response pathways that vary in accordance with their assigned category. Our category did not overlap with any know categorization (e.g. systemic-local, psychogen-somatic).
The in vivo and in vitro experiments demonstrated that the ACTH response to CRH is lower in AVP-deficient rats. This observation highlights the prominent role of AVP in ACTH secretion during acute challenges via supporting the effect of CRH. The lack of effect in di/di animals on the CRH-induced cAMP signaling demonstrates that the lower ACTH response was not due to loss of CRH signaling capacity.
The role of AVP in maintaining the basal hormonal activity of the HPA axis (ACTH and corticosterone secretion) is not supported by our results, consistently with previous reports.
The fact that the discrepancy between ACTH and corticosterone could not be explained by ACTH sensitivity or missed time points raises the possibility that other peripheral mediators are involved and may be acting directly on the adrenal gland. It supports the theory of paraadenohypophyseal neuroendocrine regulation
As a conclusion, our results support the important role of AVP in the regulation of ACTH secretion during acute stress. The requirement for AVP in acute stress regulation of HPA activity was observed in a variety of stressors and was not specified for or nor limited to a known stressor category.

Chronic stress

Repeated short periods of morphine withdrawal were associated with chronic activation of the HPA axis and increased the HPA axis activity at all three levels of the axis as it was already partly showed. Despite the predicted importance of AVP in chronic stress-induced HPA activity its absence in the Brattleboro rat did not prevent chronic stress-induced changes. On the other hand, the acute morphine injection-induced hormone rises were smaller in these rats lacking AVP. The role of AVP in opiate-induced acute HPA axis changes was supported further at the end of the chronic studies where we tested the withdrawal-induced hormone changes rather than the effect of repeated morphine administration. The lack of AVP diminished the withdrawal-induced hormone rises in the same manner as in our acute morphine injection studies.
Consistent with the assumption that CRH mRNA in the PVN is often elevated in chronic stress, we detected increased levels in morphine-dependent control (di/+) rats 16 h after the last injection. The shorter period of ‘withdrawal’ (4 h) did not induce significant changes suggesting that CRH has a regulatory role in prolonged rather than acute HPA axis changes. As the AVP-deficient rats had elevated basal CRH mRNA levels - probably due to a compensatory mechanism - we cannot conclude that the lack of elevation in this genotype was due to a regulatory role of AVP or resting levels was already at maximum and further elevation was not possible.
Gene expression of POMC in the anterior pituitary increases slowly after sustained stimulation, and thus it can reflect long-term changes as it did in morphine dependent rats. In our hand, both the 4 and 16 h withdrawal induces similar elevations and the AVP deficiency was unable to influence the basal as well as the stressed levels.
The synthesis of glucocorticoids may be enhanced after repeated stimulation. Consequently, adrenal cortex hypertrophy, elevated resting plasma corticosterone level, and thymus involution together with body weight reduction appear the best parameters of chronic stress as shown here in our study with morphine-dependent rats. Thus, we could demonstrate that intermittent morphine treatment induced a chronic stress-like state. However, we have to reject one of our hypotheses as AVP deficiency did not moderate the morphine dependence-induced changes. The di/di rats are smaller and likely to be chronically stressed even among basal conditions and it could prevent further development of chronic stress symptoms. In contrast, the lack of AVP even aggravated the thymus involution.
Our data do suggest that AVP plays a prominent role in acute morphine treatment and withdrawal-induced hormone changes without affecting the development of the chronic hyperactivity of the HPA axis, thus its role in the development of dependence is questionable.

Perinatal period

Our results demonstrate that AVP deficiency abolishes ACTH stress responses in young rats. This suggests that in these rats AVP is the key signal that controls ACTH release from the pituitary. However, corticosterone

responses were not affected by AVP deficiency. Discrepant ACTH and corticosterone responses are not explained by a differential time course of the secretion of the two hormones, and are only partly explained by the altered ACTH sensitivity of the adrenals.

The lack of ACTH stress responses in AVP-deficient pups highlights the role of AVP in the control of ACTH secretion during the perinatal period. In contrast to adult rats, where CRH is the main pituitary secretagogue, in young rats AVP is secreted in considerably larger amount.

The role of AVP appears to be limited to stress responses because basal ACTH levels were not affected by its absence. This conclusion is supported by earlier findings showing that the basal ACTH secretion is relatively independent of hypothalamic input during the stress hyporesponsive period.

The most intriguing finding of the present study is the total dissociation of ACTH and corticosterone stress responses in AVP-deficient Brattleboro pups. Normally, ACTH secretion precedes the secretion of corticosterone, and the secretion of the latter correlates with the secretion of the former. In contrast to this general picture, the ACTH response to maternal deprivation was absent in AVP-deficient rats, yet the increase in corticosterone levels was more pronounced. In pups lacking AVP, maternal deprivation increased plasma corticosterone gradually, whereas ACTH responses were absent throughout. We can hypothesized that the dissociation of ACTH and corticosterone secretion patterns was explained by the increased ACTH sensitivity of the adrenals. Because AVP might inhibit ACTH/corticosterone signal transduction, increased adrenal sensitivity may result from the removal of this inhibitory effect in di/di pups. This phenomenon may be one of the mechanisms that increase the ACTH sensitivity of the adrenals in di/di pups. Dissociated ACTH and corticosterone secretion patterns were reported in a number of earlier studies and findings suggest that the corticosterone secretion is not or weakly dependent on ACTH at early ages. Recent findings suggest that the control of corticosterone secretion has a non-ACTH component in adult animals as well. One can hypothesize that the relative importance of this component is much larger at early ages than in adulthood.

In summary, our findings show that AVP is necessary for the development of ACTH stress responses in neonates, but neither AVP nor ACTH is necessary for the development of corticosterone responses. These findings suggest that the mechanisms underlying neonatal corticosterone-stress responses are subject to debate and require further studies.

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Publications related to the thesis:

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