New hypothalamo-hypophyseal regulatory mechanisms in the pituitary hormon secretion.

Doctoral thesis

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INTRODUCTION

1. Regulation of ACTH secretion during the lactation

The hypothalamic corticotropin releasing hormone (CRH) and arginine-vasopressine (AVP) play a pivotal role in the release of adrenocorticotrophic hormone (ACTH) that is needed to maintain the internal millieu and the secretion of glucocorticoids. The hypothalamo-pituitary-adrenal (HPA) axis including its positive and negative feedback mechanisms represents ancient neuroendocrine reflexes. The hypophyseotrophic CRH neurons localized in the parvocellular compartment of the paraventricular nucleus (PVN) and their axons terminate around the capillary loops of the median eminence (ME) of the hypothalamus. Similarly, AVP is also synthetized in the PVN, but in its magnocellular part, and released into the portal vessels and reaches the anterior lobe of the pituitary by the bloodstream. The ACTH precursor molecule pro-opio-melanocortin (POMC) is expressed in the the pituitary gland, skin, immune system and the brain as well. Following translation the precursors proceeds through the Golgi-system and is stored in secretory granules where further post-translational modifications occur. The POMC is cleaved by special serin-proteases named prohormone-convertases (PC) found inside the secretory granules. PC1/3 and PC2 member of this superfamily are thought to be the principal proteases participating in the POMC maturation. Due to the different tissue expression of PCs, ACTH is dominantly synthetized in the anterior lobe (AL) and alpha melanocyte stimulating hormone (α-MSH) in the intermediate lobe (IL) of the pituitary gland. It is known that the normal cleavage process is influenced by the lack of dopamine receptor. In mice lacking D_2 dopamine receptors development of a Cushing-like syndrome has been described with melanocyte-derived hypersecretion of ACTH. Elevation of plasma corticosterone and ACTH during lactation has been well characterized since the early 1970's: The AL exhibits an impaired sensitivity to the hypothalamic CRH and AVP. This phenomenon has been supposed to be due to a "kind of chronic stress" in lactating mothers. According to our view this is not the case. For example, separation of pups from their dams can immediately cease ACTH hypersecretion. Initiation of suckling following 4 h separation of the

mothers from their pups results in a prompt rise of plasma ACTH within 15 mins.

2. Regulation of prolactin secretion

Prolactin (PRL) is a polypeptide hormone that is synthesized in and secreted from the mammotropes of the anterior lobe of the pituitary gland. In mammals PRL plays an essential role in the initiation and maintenance of lactation. Suckling, applied by the nursing pups, is the most potent physiological stimulus for PRL secretion, causes rise of blood PRL concentration within a few minutes, usually peak within 10 min and plasma levels remain elevated at a fairly high level as long as nursing continues.

The mediobasal hypothalamic dopaminergic system is the main physiological regulator of PRL secretion. The dopaminergic neurons of the periventricular and arcuate nuclei of the medial-basal hypothalamus provide dopamine to the pituitary gland. These dopaminerg neurons can be divided into three, anatomically and functionally distinct subpopulations, which are the periventriculo-hypophyseal (PHDA), a tubero-hypophyseal (THDA) and tuberoinfundibular dopaminerg (TIDA) systems. TIDA neurons terminate in the external zone of the median eminence where dopamine is released around the capillary loops and is transported through the long portal vessels to the anterior lobe (AL) of the pituitary gland. Following dopamine reaches the AL it tonically inhibits PRL secretion. THDA neurons project to the neuro-intermediate lobe of pituitary gland also play a role in the regulation of PRL secretion but together with PHDA neurons they transmit inhibition to melanocytes in the intermediate lobe.

The rate-limiting step of dopamine synthesis is the formation of L-3,4 dihydroxyphenylalanine (L-DOPA) from tyrosine. This step is catalyzed by tyrosine-hydroxylase (TH). To reach the maximal enzyme activity translated TH must undergo an important posttranslational modification, phosphorilation in the axon terminals of dopaminergic neurons. For a long time, it seemed that in contrast to the regulation of other adenohypophysial hormones, which are influenced by both inhibitory and stimulatory factors, the secretion of PRL is only under a dominant inhibitory control. It is an experimental evidence that dopamine, besides of its well-known inhibitory role, is also capable of stimulating the prolactin secretion,

especially at low concentration. In the last two decades several PRL releasing activities have also been identified.

3. Tonic inhibition through D_2-dopamin receptors

It has been recently discovered that sustained presence of the ligands of G-protein-coupled receptors (GPCRs) can promote specific intracellular signaling adaptation mechanisms parallel with the internalization process of the receptors. The receptorial desensitization or tolerance is based on these mechanisms. Desensitization has been described in the AL of pituitary gland as well, where dopamine D_2- receptors are permanently activated on lactotrophs. Ceasing of the dopaminerg inhibition is essential for the maintenance of the high secretory rate of PRL during lactation. In 2004, a new, D_2-receptor coupled signaling was described in the mouse striatum that involves the ser/thr protein kinase Akt (protein kinase B) and was used to study efficiency of psychotropic drugs and lithium. This alternative vs. "canonical" (Gi-cAMP-PKA) signaling pathway is regulated mainly by β-arrestin2, an ubiquitous molecule of receptorial desensitization. The arrestin type 1 has been associated with the dark-adaptation of the retinal cones. The GPCR-β-arrestin2-Akt-Protein Phosphatase 2A complex plays a dual role: It can terminate the G-protein-mediated signaling of the receptor and initiates a G-protein independent signaling through different downstream phosphorilation cascades e.g. Glycogene Synthase Kinase-3 (GSK3).

AIMS

1. We have investigated new regulatory mechanisms of neuroendocrine dopaminerg (NEDA) system and their possible role in PRL and ACTH secretion during lactation. Our experiments were based upon the measurement of biological responses (plasma hormone concentrations) evoked by physiological and pharmacological manipulation of D_2-dopamine receptors *in vivo*. The aims of this study were:

A. What is the the basal and suckling-induced secretory pattern of PRL, ACTH and α-MSH in lactating female rats?
B. What is the effect of dopamine D_2-receptor agonist bromocryptine (BRC), antagonist haloperidol (HAL) and

the dopamine biosynthesis inhibitor alpha-methyl-para-tyrosine (α-MpT) on the secretion of these three hormones of lactating dams, which have been separated for 4 h prior to treatments.

C. Is there any significant difference in the hormone responses between lactating, ovarectomized (OVX) and estradiol (E_2) supplemented OVX (OVX/E_2) female rats?

D. Is there any difference in the ACTH and α-MSH content (indicating changes in POMC cleavage) in tissue homogenizates prepared from the AL and IL of OVX vs. lactating female rats?

E. Does surgical disruption of the dopaminergic fibers of the pituitary stalk (PHDA, THDA) results in any change in the plasma ACTH concentration?

F. Do the suckling stimulus and the pharmacological depletion of dopamine have a synergistic or antagonistic effect on the hormone responses?

G. Does the suckling stimulus and/or pharmacological dopamine depletion have any effect on phosporilational state of TH in dopaminerg terminals of the ME?

2. Does β-arrestin dependent signaling exist within the pituitary gland?

A. Can dopamine activate the β-arrestin-dependent signaling cascade of D_2-dopamine receptor in the pituitary gland of male rats?

B. Is there a gender difference in plasma PRL levels and in the activity of β-arrestin signaling cascade following dopamine receptor inhibition?

C. What is the effect of suckling on the activity of β-arrestin dependent signaling?

D. Are the phosporilation state of both, Akt and p42/44 MAPK coupled with the suckling-induced release of PRL?

E. Is G-protein dependent or β-arrestin dependent signaling responsible for the tonic dopaminerg inhibition (tolerance and lack of dependency) of pituitary D_2-dopamine receptors?

MATERIALS AND METHODS

Primiparous lactating rats from Sprague-Dawley stock were used, which were housed in a climate- and illumination-controlled room (22±2ºC, 14: 10 h light/dark cycle) and were bred by standard rat chow.

Intravenous cannulation, suckling model and blood sampling
The suckling-induced prolactin, ACTH and α-MSH releasing effect was examined on postpartum days 7-11. Two days prior to experiment, a permanent cannula was implanted into the jugular vein under ether anaesthesia, allowing frequent blood sampling from freely moving rats. On the day of the experiment, following 4 hours separation period, blood samples were taken 15, 30, 60, 75 and 90 after the pups reunion or pharmacological treatment.

Radioimmunoassay
Plasma PRL, ACTH and α-MSH were measured by RIA The measurement of plasma hormone concentration was completed by neuro-intermediate lobe (NIL) and AL hormone level assay (ACTH, α-MSH).

Pharmacological treatments
1. Pharmacological manipulation of the NEDA system in lactating, ovariectomized and estradiol-substituted rats:

a. The extracellular dopamine-depletion were done by a tyrosine-hydroxylase inhibitor, alpha-methyl-p-tyrosine (25 mg/kg iv.) following 4 hours separation period or

b. following 4 hours separation we administered *peripheric* D_2-dopamine receptor antagonist, domperidone (25 μg/rat iv.).

c. For answering the question, whether the dopamine agonist pretreatment is able to prevent the suckling induced PRL and ACTH response, we administered bromocryptine (3 mg/kg ip) 60 min before suckling.

2. *Common effect of suckling and TH inhibition on plasma PRL, ACTH, α-MSH levels:*

a. Following 4 hours separation dams were treated an intravenous injection of αMpT (8 mg/kg) via jugular vein cannula implanted the previous day and the pups were returned to the mothers 60 min after the injection.

b. Following 4 hours separation the pups were returned to the dams and after blood sample taking at 60 min of reunion mothers were administered an intravenous injection of αMpT (8 mg/kg).

3. For the immunohistochemical investigation of phosphorylated TH

3 groups were used:
- αMpT injection (200 mg/kg ip.) 30 min prior to 4 hours separation period
- continuously suckled
- or separated (for 4 hours) dams.

4. Other pharmacological treatments used in experiments (western-blot and icv injection types):

Ocadaic acid (0,5 µl, 0,5 mM solved in 20% DMSO, icv)

SL327, selective MEK1/2 (MAP-kinase-kinase) inhibitor (5 mg/kg ip.)

Haloperidol (HAL), selective D_2-receptor antagonist, 2,5 mg/kg ip.

Raclopride (RAC), selective D_2-receptor antagonist, 2,5 mg/kg ip.

Posterior pituitary denervation:
To prove that THDA and PHDA neurons take part in the alternative splicing of POMC in the intermediate lobe during lactation we performed the posterior pituitary denervation also in lactating rats and measured the plasma level of ACTH.

Immunostaining
In the median eminence of the hypothalamus TH and phosphoTH immunoreactive structures were visualized by using the appropriate polyclonal antibodies, the ABC technique and Ni-DAB exposition. The regional optic densitometry of the microphotographs was performed using ImageJ Image Process and Analysis Software.

Western-blot
Lactating mothers were decapitated and the anterior lobes of the pituitary were removed and homogenizated 10 and 60 min after starting the suckling stimulus together with trunk blood collection. In males we removed organs 30, 60 and 120 min after pharmacological treatments (dopamine antagonists). From bilaterally ovariectomized rats pituitaries were removed 60 min after haloperidol injection.
To decide whether ERK-phosphorylation plays a role in the control of suckling-induced PRL release, we administered intraperitoneal MAP-kinase-kinase inhibitor SL327 30 min prior to suckling. We used the following commercial antisera: Cell Signaling Technology anti-total-Akt, anti-phospho-Akt (Thr308), anti-total-p42/44 MAP-kinase, anti-p42/44 phospho-MAP-kinase and anti-β-aktin.

Catecholamine measurement with HPLC
From anterior- and neurointermediate lobe homogenizates prepared from lactating vs. OVX rats dopamine and DOPAC concentration was measured by using C18 reverse-phase column HPLC.

Intracerebro-ventricular microinjection
Protein phosphatase 2A (PP2A) inhibitor, ocadaic acid was injected into the third vetricule via stereotaxically implanted cannula and changes of plasma PRL level was determined.

RESULTS

1. Comparison of plasma and hypophyseal hormone concentrations between lactating and OVX female rats following physiological and pharmacological manipulation of NEDA neurons.

Plasma concentrations of ACTH and PRL increased in response to the suckling stimulus. Pretreatment of rat mothers with the D_2-receptor agonist BRC completely prevented the ACTH and PRL secretory responses to the suckling stimulus. In lactating animals pharmacological dopamine depletion significantly increased the plasma ACTH concentration, in contrast to OVX and E_2-substitued OVX females in which dopamine depletion did not elevate the ACTH release. Dopamine depletion significantly enhanced PRL secretion in every experimental groups but it had no influence on plasma α-MSH level in the lactating group. BRC pretreatment

blocked both plasma PRL and ACTH responses evoked with dopamine antagonists.

Using suckling stimulus and dopamine depletion together we have demonstrated, that suckling stimulus raised PRL and ACTH plasma levels already at 15 min, but α-MpT injected at 60 min did not cause more significant elevation.
In contrast α-MpT administration prior to suckling stimulus significantly increased plasma PRL and ACTH concentration, but suckling stimulus started 60 min following the injection did not cause more changes.

ACTH and α-MSH levels in tissue were also measured by RIA in lactating and OVX female rats:

A. ACTH concentration of the NIL was about two times higher in lactating rats than in OVX rats.
B. The ACTH concentration of the AL was three times higher in OVX than in lactating rats.
C. The α-MSH concentration of the NIL was significantly lower in lactating dams than in OVX females.
D. The α-MSH level of the AL was 8 times higher in lactating dams than in OVX females.

According to the HPLC measurement of DA and DOPAC the DA content was about 8-fold lower in the IL of lactating mothers compared to OVX female rats.

Using posterior pituitary denervation we could demonstrate that hypophyseal AVP (produced by posterior pituitay) does not play a role in the control of ACTH release.

2. Functional morphology of TIDA neurons following suckling stimulus and/or pharmacological depletion of hypothalamic dopamine.

After 4 h separation of the lactating mothers from their litters phospho TH-immunoreactivity increased in the external zone of mediane eminence compared to continuously suckling or α-MpT

pretreated dams. In continuously suckled rats, low number of pTH-immunoreactive perikarya or terminals can be found in this region.

3. Significance of new signaling pathways in the anterior lobe of the pituitary gland

In males both HAL and αMpT administration increased phosphorilation of Akt and p42/44 MAP-kinase (ERK) in the AL of pituitary.
We have studied the time course of phosphorilation and we concluded that the highest level of Akt phosphorilation could be found 60 min after pharmacological dopamine depletion done by HAL injection in males. HAL and RAC proved to be equivalent in their effect to phosphorilation.
Using different suckling time periods we have demonstrated that the highest level of phosphorilated Akt and MAPK can be detected at the 10th min of suckling in the AL of pituitary gland.
Pharmacological depletion of dopamine elevated the level of phospho-Akt in all experimental groups (male, OVX and lactating females).
Suckling significantly augmented MAPK phosphorilation, but after inhibition of phosphorilation of MAPK did not affect the suckling-induced plasma PRL change.

DISCUSSION

We can conclude that the pattern of pituitary PRL, ACTH and α-MSH secretion is different during the lactation period compared to other physiological states. This is likely due to an altered responsiveness of the dopaminerg systems and/or the difference of the POMC processing in the pituitary AL and IL.
Subpopulations of NEDA neurons projecting to the IL (THDA and PHDA) are associated primarily with the control of α-MSH secretion. In lactating females they may play a role in the regulation of ACTH secretion, therefore securing the higher hormone level independently from the HPA axis. Since the pharmacological withdrawal of dopamine has resulted in a clear difference in the ACTH secretion between OVX and lactating group and surgical deafferentation of the posterior pituitary also elevated basal levels of ACTH in the lactating group, it seems very likely that hypothalamic NEDA neurons play a significant and previously unrecognised role

in the regulation of ACTH secretion during lactation. The ACTH, detected in the plasma of lactating dams, may appear to originate partially from the IL. Furthermore, differences in ACTH and α-MSH responses between OVX and lactating females indicate the existence of a physiologically important plasticity of melanotropes in the IL of the pituitary gland.
Furthermore, our findings, obtained by using phosphor-Ser40 specific TH antibody, is the first functional neuromorphological observation, which clearly shows that the level of phosphorylation i.e. activation of TH in the ME region of the hypothalamus (TIDA neurons) is closely related with the plasma level of PRL and ACTH in lactating rats.

It has been demonstrated first time that physiological and pharmacological manipulation of NEDA neurons can result in a definitive change of dopamin-mediated signaling based on the state of the arrestin-Akt-PP2A complex: pharmacological depletion of dopamin parallel with the plasma PRL response resulted in a distinct increase in the phosphorilation states of Akt in the AL of pituitary and the importance of the cAMP-mediated signaling is diminished. However the upstream and downstream elements of the cascade and the exact mechanism of activation are not fully cleared in pituitary yet, the involvement of this signaling in the anti-apoptotic regulation is undoubtful.
Systematic, intraperitoneal administration of D_2-antagonist haloperidol and raclopride has elevated the level of phospho-Akt in male, ovarectomized as well as in lactating female rats indicating the uncoupling of the arrestin-Akt-PP2A, so called β-arrestin-dependent signaling complex. Since the suckling stimulus resulted in a similar phenomenon, we can conclude that the changes in this alternative signaling pathway should be major contribution in the ceasing of tonic dopaminerg inhibition.

PUBLICATIONS RELATED TO THE DISSERTATION

List of own publications related to the topic of the doctoral thesis

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