

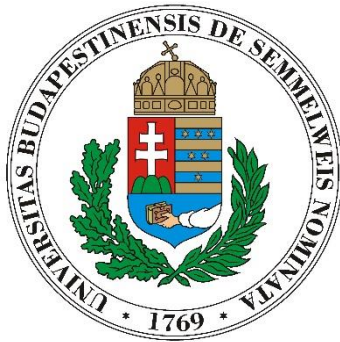
Role of Insulin-like growth factor-1 (IGF-1) in maternal adaptation of the central nervous system

Doctoral thesis

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I. INTRODUCTION

Postpartum physiological and behavioural changes are important parts of mammalian reproduction, and they can be investigated using the rat as an animal model. Non-maternal females do not care about pups or even attack them, while mothers demonstrate well-defined maternal behaviours, e.g., nest building, pup retrieval to the nest, nursing, and decreased anxiety, in addition to lactation. These marked changes are the consequences of maternal adaptation of the central nervous system. Prolactin and other hormones contribute to the initiation of maternal behaviours, but are not required for it. Rather, maternal behaviour is controlled by a complex neuronal network in which the medial preoptic area (MPOA) plays a central role. The density of active neurons is dramatically induced in the MPOA of parenting females. Furthermore, lesions of the MPOA abolish the nest building and retrieving components of maternal behaviour in lactating females, while electrical and optogenetic stimulation of this area enhances maternal responsiveness.

After parturition, not only maternal behavior appears, but lactation also. Higher prolactin (PRL) secretion from the pituitary and concordant lactation is one of the most important postpartum physiological changes, which requires the adaptation of central nervous system. PRL facilitates the adaptation of maternal central nervous system and whole organism to the postpartum state. PRL is a polypeptide hormone, secreted mainly by the lactotroph cells of the anterior pituitary. These cells are under a tonic inhibition by dopamine secreted from the tuberoinfundibular dopaminergic (TIDA) neurons of the arcuate nucleus. Higher PRL secretion could be reached only by disinhibition. These TIDA neurons are located on both sides of third ventricle, in the dorsomedial subdivision of arcuate nucleus. PRL develops a negative feedback on TIDA neurons, which is the only feedback mechanism in the hypothalamo-prolactin axis. TIDA cells express PRL receptor and PRL enhances expression of tyrosine-hydroxylase enzyme (TH), dopamine metabolism in the median eminence and dopamine secretion into portal blood vessels. Effect on TH is important, because this enzyme performs the first and rate-limiting step of catecholamine synthesis. Amount of TH is regulated mainly by its expression and activity by its phosphorylation. Rat TH enzyme could be phosphorylated at 4 different sites: Ser8, 19, 31 and 40. Phosphorylation at Ser31 and 40 elevates enzyme activity and stability. Ser31 used to be phosphorylated by ERK1/2 and Cdk5

(cyclin dependent kinase 5) and Ser 40 by PKA and MAPK (mitogen activated protein kinase).

Insulin-like growth factor-1 (IGF-1) is an evolutionary ancient peptide and its role in supporting cell survival, proliferation and differentiation is widely known. Circulating IGF-1 is mainly produced and sequestered by the liver under the control of growth hormone (GH). Therefore IGF-1 is the main mediator of GH actions, although it is expressed in the central nervous system and peripheral tissues where it acts as an autocrine or paracrine factor. IGF-1 is important in the connection of GH and other neuroendocrine, such as the hypothalamo-pituitary-gonadal and hypothalamo-prolactin-lactation axis. GH is suggested to play an important role in the maintenance of lactation in the rat: milk yield is reduced by approximately 50% in the absence of PRL and milk yield is totally stopped in the absence of PRL and GH. GH is necessary for the synthesis of milk with normal composition and enhances milk production in human, too. IGF-1 induces prolactin secretion from the pituitary and milk production in the mammary gland.

The actions of IGF-1 are mediated by a cell surface receptor, type 1 IGF receptor (IGF-1R), which is the major transducer of IGF signals. Insulin-like growth factor binding protein-3 (IGFBP-3) binds insulin-like growth factor-I (IGF-1) in the plasma and extracellular space. Although 6 other IGFBPs exist, IGFBP-3 is the major carrier of IGF and binds the majority (75-90%) of IGF-1 in circulation. By binding IGF with high affinity in a functionally inactive complex, IGFBP-3 can inhibit the effects of IGF-1 on the IGF-1R.

The above mentioned MPOA is a key player in the regulation of maternal adaptation with remarkable alterations in gene expression levels postpartum. The hypothesis of our laboratory was during a previous study that genes with altered maternal expression could play a role in central maternal adaptations. Our laboratory performed a microarray study of the preoptic area to identify which genes show markedly change in lactating mothers compared to pup-deprived mothers, as a first step of confirming the hypothesis. The pups of pup-deprived mothers were taken away right after delivery, therefore maternal adaptation did not occur. These mothers did not lactate and did not show any component of maternal behavior. We re-evaluated the results of the microarray and compared it to another study. We identified 21 genes with highly significant differences in expression levels between the maternal and pup-deprived groups ($p < 0.007$) in our previous experiment. Islet amyloid polypeptide (amylin)

exhibited the greatest elevation in mothers compared with that in pup-deprived controls and has been identified as a novel neuropeptide with maternal functions in the rat. The expression levels of 5 other genes were 2-4 times higher in rat dams than those in the pup-deprived control group. Of these genes, IGFBP-3 was also up-regulated in the other study. Therefore, we choose to further investigate the maternal function of this protein and IGF-1. ***Our hypothesis was that the IGF-system (IGF-1 and IGFBP-3) plays a role in maternal adaptation of the central nervous system.***

II. AIMS OF THE STUDY

1. Validation of the induction of IGFBP-3 in the preoptic area of rat mothers

- a.) Could be verified the maternal induction of IGFBP-3 in the preoptic area with quantitative RT-PCR?
- b.) Where does the maternally elevated expression of IGFBP-3 localize in the dissected preoptic area?

2. Effect of IGF-1 on maternal motivation

- a.) Does the icv. administration of IGF-1 or an IGFBP-3 inhibitor affect maternal motivation?
- b.) Are the IGF-induced alterations of maternal motivation coherent with the differences in anxiety-like behavior and general motor activity?

3. Effect of IGF-1 on lactation

- a.) Does the chronic IGF-1 treatment affect suckling-induced prolactin release?
- b.) Does the important member of IGF-system, IGFBP-3, express in the prolactin secretion regulating TIDA neurons?

Does the expression of IGFBP-3 change according to lactation in the TIDA neurons?

How does the IGF-1 affect expression of TH enzyme *in vivo* and *in vitro*?

How does the IGF-1 influence activity affecting phosphorylation of TH enzyme *in vitro*?

4. Alterations of circulating IGF-1 level according to lactation

- a.) How does the serum IGF-1 concentration change during suckling in rat dams?

Is there any connection between prolactin and IGF-1 levels during suckling?

- b.) How does the acute or chronic icv. administration of IGF-1 affect circulating IGF-1 level during suckling?

III. METHODS

Our animal experiments were conducted in the Department of Anatomy, Histology and Embriology of the Semmelweis University, under the permission No. 44009/2013 of the Government Office for Pest County, Directory of Food Chain Security and Animal Health.

We dissected the preoptic area from lactating and pup-deprived mothers for RT-PCR to validate the more than twofold elevation of IGFBP-3 mRNA expression in the previous microarray study. The maternally induced IGFBP-3 expression was localized using in situ hybridization histochemistry (ISHH) in the preoptic area.

We administered icv. IGF-1, an IGFBP-3 non-peptide ligand inhibitor (NBI-31772) and artificial cerebrospinal fluid (ACSF) as a control to rat dams 14 days long after delivery, through implanted brain infusion kits connected to osmotic minipumps. We investigated the behavioral effects of the prolonged icv. administration, performing pup-retrieval test (on the 6th day postpartum), elevated plus maze test (on the 7th day postpartum) and observing undisturbed maternal behavior for 5 days, three times per day (between 4-9th day postpartum).

We used brain infusion kits connected to osmotic minipumps for examining the effects of IGF-1 on prolactin secretion. We obtained blood from rat dams during suckling through jugular cannula, on the 14th day postpartum after a 12 days long constant IGF-1 treatment to investigate the suckling induced prolactin secretion. To discover the potential mechanisms in the background of IGF-1 effect, we carried out an IGFBP-3 ISHH and combined it with TH immunohistochemistry in the arcuate nucleus, focusing on the prolactin secretion regulator TIDA neurons. In addition, we investigated the TH expression in this area using ISHH in IGF-1 treated and control mothers. We analyzed the effects of IGF-1 on TH expression and phosphorylation in vitro, in primary mediobasal hypothalamic cell cultures, using RT-PCR and Western Blot techniques. We performed the Western Blot with phosphorylation site specific antibodies.

We described the alterations of serum IGF-1 level during suckling, using ELISA of blood samples from lactating rat dams. We obtained blood through jugular cannula during suckling. We used prolonged icv. IGF-1 treatment with osmotic minipumps and rapid administration through icv. cannula to discover the potential regulatory mechanisms in the background. We investigated the effect of these manipulations on the serum IGF-1 levels during suckling.

IV. RESULTS

1. Validation of the induction of IGFBP-3 in the preoptic area of rat mothers

Because of the high rate of false positive data in microarray experiments, we measured the mRNA level of IGFBP-3 in rat dams and pup-deprived controls with 2 independent methods, RT-PCR and in situ hybridization histochemistry, as IGFBP-3 induction has not been validated in the previous studies. Both methods validated the increased IGFBP-3 expression level in the preoptic area in lactating rat dams. The confirmed maternal elevation in IGFBP-3 expression and its distribution in the region of the preoptic area involved in maternal behavior, the medial preoptic area, suggest its involvement in the control of maternal behavior. The IGFBP-3 expressing neurons were localized with highest density in the central subdivision of the medial preoptic nucleus (MPN). The number of active neurons raise markedly not only after delivery, but also in virgin females in the presence of pups. Therefore we can hypothesize that elevated IGFBP-3 expression plays role in the maternal adaptation of central nervous system.

2. IGF-system in the regulation of maternal responsiveness

IGFBP-3 binds IGF-1 with high affinity, thereby reducing the level of bioavailable IGF-1. To examine the role of IGFBP-3 in the regulation of maternal behaviors and lactation, we inhibited this IGF-sequestering effect with continuous and prolonged icv. infusion of either IGF-1 or an IGFBP ligand-binding inhibitor, NBI-31772. These infusions presumably increased the preoptic bioavailability of IGF-1 and neutralized the maternally elevated IGFBP-3. The mothers showed several aspects of normal behavior, demonstrating that they can perform maternal behaviors. However, we measured an increased latency to carry the first pup and the last pup into the nest following the sequestration of IGFBP-3. Since moving the pups into the nest is the most frequently used indicator of maternal motivation as a goal-directed behavior, our results suggest that elevated expression of IGFBP-3 in the MPOA in lactating dams contributes to maternal motivation. Different factors are known to contribute to the initiation and maintenance of maternal behavior. While peripartum hormonal changes initiate and potentiate the onset of maternal motivation, they are not required for maintenance of maternal care. Continuous exposure to pups and afferent stimuli from them are sufficient for supporting the maintenance of maternal motivation during the postpartum period. The neurochemical regulatory mechanisms, which contribute to ongoing maternal care, are much

less established. Based on our results, IGF-1 and IGFBP-3 appear to contribute to the maintenance of maternal behaviors, thereby suggesting a previously unknown mechanism involved in maternal regulations. The lack of effects of prolonged IGF-1 and NBI-31772 on activity in the elevated plus-maze test indicates that reduced maternal motivation was not a consequence of altered state of anxiety, which itself could affect maternal behavior.

3. IGF-system in the regulation of lactation

In rodent mothers, lactation is driven by the suckling pup stimuli, which evokes a dramatic increase in serum prolactin levels. IGF-1 and ACSF as control was continuously administered *icv. via* osmotic minipumps implanted in rat mothers on the 2nd postpartum day. The serum prolactin levels induced by suckling were measured on the 14th postpartum day. We observed a high degree of elevation in control mothers, which received ACSF injection. However, prolactin levels increased to a significantly lesser degree after the administration of IGF-1, supporting a role of IGF-1 in the regulation of prolactin secretion. At the same time, as prolactin levels were reduced in the presence of IGF-1, the body weight of pups gained during 1 hour of suckling was also decreased in the IGF-1 group compared with that in the controls. These are new findings as hypothalamic IGF-1 have not been shown to control prolactin secretion in mothers.

Hereunder we investigated, if TIDA neurons, which regulate prolactin secretion on the hypothalamic level, express IGFBP-3 and if the expression of IGFBP-3 is dependent on lactation, like in the MPOA. We combined IGFBP-3 *in situ* hybridization histochemistry with TH immunohistochemistry to determine if dopaminergic neurons express IGFBP-3 mRNA in the arcuate nucleus. We found a high degree of co-localization; 67.3% of TIDA neurons contained IGFBP-3 and 85.9% of IGFBP-3 neurons contained TH immunoreactivity, suggesting that in the arcuate nucleus of lactating mothers, IGFBP-3 mRNA expression is located in TH-immunoreactive dopaminergic neurons. We described IGFBP-3 expression in the paraventricular nucleus (PVN) in addition to the arcuate nucleus. The quantification of the *in situ* hybridization histochemistry signal revealed that IGFBP-3 had an approximately 3-fold increase in the expression level in the dorsomedial subdivision of the arcuate nucleus in mother rats compared with that in pup-deprived controls. In contrast, there was no maternal increase in the expression level of IGFBP-3 in the PVN. In conclusion, IGFBP-3 expression is higher in TIDA neurons during lactation, likely to neutralize the prolactin secretion inhibiting effects of IGF-1.

Binding of IGF-1 to its receptor results in autophosphorylation of the receptor, which in turn initiates a cascade of cellular signal transduction events. One key step is the binding of insulin receptor substrate (IRS)-1 to phosphotyrosine residues on the receptor. IRS-1 then acts as a docking protein for the downstream signal transduction components, including the Ras/ERK1/2 and PI3K/Akt pathways. IGF-1 supports the survival of dopaminergic neurons through these two pathways, which has mostly been investigated in the nigrostriatal dopamine system. In line with these data, IGF-1 enhanced TH expression in our hypothalamic cell culture experiments and in lactating rat dams after prolonged icv. administration. In dopaminergic neurons, activated ERK1/2 proteins enhance the transcriptional activation of TH by nuclear receptor related-1 (Nurr1) which therefore represents a possible mechanism of the effect of IGF-1 on TIDA neurons in our study. In addition, we also found an increase in the level of phosphorylation of the TH enzyme at the Ser 31 phosphorylation site by IGF-1 in vitro. This finding suggests rapid enhancement of dopamine synthesis by IGF-1 as phosphorylation of TH results in an increased catecholamine production. This rapid action of IGF-1 on dopaminergic neurons has not been previously described. ERK1/2 serine kinases are mainly responsible for Ser 31 phosphorylation suggesting that IGF-1 may have acted via ERK1/2 serine kinases to induce phosphorylation of TH.

4. Alterations of serum IGF-1 level according to lactation

We investigated in our further experiments, how serum IGF-1 concentration changes during suckling. IGF-1 can cross the blood-brain barrier and act in the hypothalamus. This effect can be modulated by hypothalamic produced IGFBP-3. We established already that IGF-1 lowers suckling-evoked prolactin release by inducing the TIDA neurons and this effect of IGF-1 should be neutralized. Therefore, our hypothesis was that elevated IGFBP-3 expression in lactating mothers is important, because level of IGF-1 alternates during suckling. In line with our hypothesis, we could describe significant suckling-induced serum IGF-1 elevation, which was established at 30 min after the beginning of suckling and calmed down at 60 min, as a major novel finding. The time course of increase in IGF-1 level was similar to the increase in GH and in PRL levels in response to suckling. In addition, during the suckling period we found a significant correlation between serum PRL and IGF-1 levels, which strengthens that it might be an association between these two hormones. We assume, that the most probable source of the rapid increase of serum IGF-1 level might be its secretion from the liver, because this organ contributes around 70% of the total circulating IGF-1. Suckling can stimulate IGF-1 either via GH or PRL. On the one hand, suckling-induced GH

release can induce IGF-1 release from the liver. On the other hand, PRL can increase circulating IGF-1 levels acting directly within the liver.

We investigated possible regulatory mechanisms in the background of suckling-induced IGF-release. Chronic icv. IGF-administration delayed the IGF-surge as suckling did not cause any induction in circulating IGF-1 levels after 30 minutes in the presence of exogenous supplied IGF-1. Although there was a significant increase after 60 minutes, but our data are not sufficient to establish it firmly whether it is a surge or not because we do not have information on later time points. Central IGF-1 can exert this effect in the hypothalamus either by acting as a long-loop negative feedback modulator of GH axis or by suppressing suckling-induced PRL release. IGF-1 is responsible for the long-loop negative feedback in the GH-IGF axis and we established, as I mentioned above that prolonged icv. administration of IGF-1 in the postpartum period suppresses suckling-induced PRL release.

Acute IGF-1 injection 5 minutes prior to the beginning of suckling did not have any effect. As we described above, effects of IGF on PRL release and long-loop negative feedback of GH axis likely acts on the gene expressional level, hence acute injection could not have any impact. Although there was a peak at 30 minutes in suckling-induced IGF-release in the continuously ACSF treated control group, following acute injections, IGF-1 level remained high at 60 minutes, too, possibly due to stress-related effects. PRL serum concentration also remained high and this elevated PRL could sustain the high level of IGF-1, even after 60 minutes. This difference of the pattern could be due to the effect of stressful acute manipulations of the cerebrospinal fluid (CSF) *via* icv. injection, because stress could cause an additional induction of PRL release.

In conclusion, suckling is able to induce the elevation of serum IGF-1 concentration via enhancing either prolactin or GH secretion. This effect of suckling can be inhibited by central, chronic administration of IGF-1 either via affecting dopamine synthesis of TIDA neurons or exerting negative feedback on GH. The suckling-induced IGF-1 release may have a physiological importance because circulating IGF-1 promotes lactation via different ways: facilitating PRL release from the pituitary, increasing milk synthesis in association with mammary blood flow and inhibiting mammary cell apoptosis during late lactation. On the other hand, higher IGF-1 level in the hypothalamus would induce TH expression and activation by phosphorylation in TIDA neurons leading to an increased dopamine secretion and concomitant prolactin suppression. To neutralize this effect, IGFBP-3 expression elevates during lactation in TIDA neurons. This hypothesis represents a novel mechanism in

neuroendocrinology; neurons can block the effects of a hormone by inducing the expression of its binding protein.

V. CONCLUSION

1. IGFBP-3 expression is elevated in the medial preoptic area and dorsomedial arcuate nucleus of lactating rat mothers. We validated with RT-PCR the preoptic microarray results and localized the elevated IGFBP-3 expression with ISHH to the medial preoptic nucleus and TIDA neurons of the arcuate nucleus.
2. Prolonged central administration of IGF-1 and an IGFBP-3 antagonist lengthened the pup-retrieval time which means it decreased maternal motivation. The effect was specific on maternal motivation, because it did not affect undisturbed maternal, anxiety-like behavior and general activity.
3. Prolonged IGF-1 treatment decreased suckling-induced prolactin release.
4. IGF-1 induces the expression of TH enzyme in TIDA neurons *in vitro* and *in vivo*. In addition, it enhances activating phosphorylation of the enzyme at Ser31 *in vitro*.
5. As a novel finding, we could describe the suckling-induced IGF-1 secretion. The serum IGF-1 level shows a markedly elevation from the beginning of suckling, reaches its maximum at 30 min, significantly correlating with prolactin level. Prolonged central IGF-1 treatment diminished this induction. On the other hand, acute IGF-1 administration, directly before suckling, did not have any effect neither on prolactin nor on IGF-1 secretion.

VI. PUBLICATIONS

1. Publications in the topic of the thesis

Leko AH, Cservenak M, Szabo ER, Hanics J, Alpár A, Dobolyi A (2017) Insulin-like growth factor I and its binding protein-3 are regulators of lactation and maternal responsiveness. *Sci Rep.* 7:3396. **IF: 4,259**

Lékó AH, Cservenák M, Dobolyi Á. (2017) Suckling induced insulin-like growth factor-1 (IGF-1) release in mother rats. *Growth Horm IGF Res.* 37:7-12 **IF: 1,828**

2. Other publications

Cservenák M, Szabó ÉR, Bodnár I, **Lékó A**, Palkovits M, Nagy GM, Usdin TB, Dobolyi A. (2013) Thalamic neuropeptide mediating the effects of nursing on lactation and maternal motivation. *Psychoneuroendocrinology* 38:3070-3084 **IF: 5.591**

Cservenák M, Keller D, Kis V, Fazekas EA, Öllös H, **Lékó AH**, Szabó ÉR, Renner É, Usdin TB, Palkovits M, Dobolyi Á. (2017) A thalamo-hypothalamic pathway that activates oxytocin neurons in social contexts in female rats. *Endocrinology* 158(2):335-348 **IF: 4,286**

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