

**The role of tuberoinfundibular peptide of 39 residues and
parathyroid hormone 2 receptor neuromodulator system in
the central regulation of maternal adaptations**

Ph.D. thesis

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I. INTRODUCTION

Tuberoinfundibular peptide of 39 residues (TIP39), purified from bovine hypothalamus, is the only known endogenous high-affinity ligand of the PTH-2 receptor (PTH2 receptor). TIP39-expressing cells in the brain are restricted to the subparafascicular area of the thalamus and the medial paralemniscal nucleus of the pons (MPL). The subparafascicular area TIP39 neurons can be subdivided into a medially located group in the periventricular gray of the thalamus (PVG) and a laterally positioned group in the posterior intralaminar complex of the thalamus (PIL).

The PTH2 receptor was identified by Ted B. Usdin and his coworkers in 1995. Activation of PTH2 receptor by TIP39 results in the accumulation of cAMP and Ca^{2+} . In contrast to the TIP39 neurons, PTH2 receptor expressing neurons are widely distributed in the central nervous system: dense PTH2 receptor expression was found in limbic structures, the hypothalamus, certain areas of the thalamus, brainstem and even in the spinal cord.

A marked decrease was observed in the level of TIP39, but not the PTH2 receptor expression during the period of pubertal development.

The physiological role of the TIP39-PTH2 receptor system is not well understood yet. According to earlier studies, TIP39 neurons mediate stress response to auditory stimuli. Intracerebroventricular administration of TIP39 leads to increased tolerance of nociceptive stimuli, increase of corticotropin-releasing hormone (CRH) and inhibition of growth hormone (GH) release.

For all mammals, successfully rearing offspring entails the recruitment of a wide array of behavioral and physiological adaptations. The adaptive changes of pregnancy are the consequence of signals from the

conceptus, in large part as hormones, but partly arising from the physiological changes occurring during pregnancy while postpartum, the signals result from the interactions between the offspring and the mother, and neuroendocrine mechanisms. In this respect, it is the developing offspring that determines, especially through the suckling stimulus, much of what happens in the mother's brain.

The most conspicuous endocrine event in the postpartum period is lactogenesis/galactopoiesis. The suckling induced prolactin release is the neuroendocrine reflex, which is responsible for milk production. Prolactin (PRL) secretion and release by mammatropes in lactating rats are mainly controlled by dopaminergic neurons of the medial basal hypothalamus. Dopamine tonically inhibits prolactin production and release in non-lactating rats. At the beginning of lactation, suckling stimuli by the pups reach the hypothalamus and inhibit the activity of tuberoinfundibular (TIDA) neurons, thus allowing the release of PRL from the pituitary into the circulation and in turn, prolactin stimulates milk secretion.

Jay Rosenblatt's approach-avoidance model of maternal behavior proposes that maternal behavior occurs when the tendency to approach infant stimuli is greater than the tendency to avoid such stimuli. The maternal motivational system – especially the medial preoptic area (MPOA) and the ventral part of the bed nucleus of the stria terminalis (vBNST) - is responsive to hormones and pup stimuli. The output of this system is elevated attention and attraction of female toward their pups. It produces this effect by depressing competing motivational systems, such as those regulating defensive behavior and avoidance, while at the same time increasing the tendency to respond maternally to pups.

II. OBJECTIVES

1. Description of the topography of TIP39 neurons in the PIL and their neuronal connectivity in mother rats according to the points below:

- A. What sort of markers exist in neurons of the PIL and how do they relate with TIP39 neurons?
- B. Where do the TIP39 neurons of the PIL project?
 - B/1. Do TIP39 fibers disappear from the hypothalamus due to the electrical lesion of the PIL?
 - B/2. Where do TIP39 fibers arising from PIL run?
 - B/3. What kind of projection pattern do the neurons have in the PIL?
- C. What kind of afferent neuronal connections do the PIL possess?

2. Investigation of induction of TIP39 and activation of TIP39 neurons in mother rats. Our questions were the following:

- A. Do TIP39 mRNA levels change during pregnancy and lactation?
- B. Does TIP39 immunoreactivity change during lactation?
- C. Which effects influence the activation of TIP39 neurons?

3. Examination of the effects of antagonism of endogenous TIP39 on suckling induced prolactin release. We addressed the further questions:

- A. Does intracerebroventricular injection of the PTH2 receptor antagonist (HYWH-TIP39) affect suckling induced prolactin release?
- B. Does the mediobasal hypothalamic injection of HYWH-TIP39-GFP-expressing lentivirus vector have an effect on suckling induced prolactin release?

4. Examination of the effects of antagonism of endogenous TIP39 on maternal motivation:

A. Does the medial preoptic injection of HYWH-TIP39-GFP-expressing lentivirus vector affect maternal motivation?

III. METHODS

Experiments were carried out according to protocols approved by the Animal Examination Ethical Council of the Animal Protection Advisory Board at the Semmelweis University, Budapest, and meet the guidelines of the Animal Hygiene and Food Control Department, Ministry of Agriculture, Hungary.

Animal experiments and histochemical studies were performed in the Department of Anatomy, Histology and Embryology of the Semmelweis University.

We used Luxol fast blue staining to describe the topography of PIL and fluorescent histochemistry to describe the presence of neurochemical markers in the PIL and their relations with TIP39 neurons.

We investigated the neuronal connectivity of TIP39 neurons in the PIL using transection and electrical lesion techniques, as well as neuronal tracers (cholera toxin β -subunit, biotinylated dextran amine).

We examined the TIP39 induction in mother rats using immunohistochemistry, *in situ* hybridization and RT-PCR.

Two different methods were used to detect the effect of endogenous TIP39 antagonism on suckled induced prolactin release. First, we injected a PTH2 receptor antagonist (HYWH-TIP39) into the lateral ventricle. Then we injected a PTH2 receptor antagonist expressing-virus (HYWH-TIP39-GFP) to the mediobasal hypothalamus. We took blood

samples from mothers through jugular cannulae and assigned prolactin concentrations with radioimmunoassay.

Finally, we analyzed the effects of medial preoptic injection of HYWH-TIP39-GFP lentivirus on maternal motivation with conditioned place preference test.

IV. RESULTS

1. TIP39 neurons in the posterior intralaminar complex of the thalamus

The PIL defined by the area containing TIP39 neurons includes the posterior intralaminar thalamic nucleus, the parvicellular subparafascicular nucleus and some parts of the caudal subdivision of the zona incerta.

The PIL contains a high density of CB-ir cell bodies as does the triangular subdivision of the posterior thalamic nucleus immediately dorsal to the PIL. Within the PIL, both TIP- and CB-ir cell bodies are evenly distributed. Furthermore, almost all TIP39-ir neurons contain CB immunoreactivity.

Coolen and his coworkers subdivided the PIL on the basis of results using different neurochemical markers. The medial subdivision contains galanin-immunoreactive axons, which originate from the lumbosacral spinal cord. In turn, the lateral subdivision includes calcitonin-gene related peptide (CGRP)-ir neurons and fibers. The calbindin-ir neurons and the substance-P fibers are present in both of them. In addition, matting-induced neuronal activation was revealed only in the medial subdivision.

We observed the PIL TIP39 neurons located medial to CGRP neurons. They overlap with galanin-fibers and show c-fos activation in the

course of ejaculation in male rats, so we classified them to the medial subdivision.

Connections of the TIP39 neurons in the PIL remained to be elucidated before our work. Thus, the projections of the neurons in the PIL established in this study provide the anatomical basis of how a variety of limbic structures such as the medial prefrontal cortex, the accumbens nucleus, the lateral septum, the fundus striati, the periaqueductal grey as well as hypothalamic structures including the medial preoptic area, the paraventricular and periventricular hypothalamic nuclei, the arcuate nucleus can be influenced by PIL neurons. After injection of anterograde neuronal tracer to the PIL, the anterogradely labeled fibers from the PIL join the supraoptic decussations and run towards the hypothalamus. After unilateral surgical transection of the supraoptic decussations in mother rats, accumulation of TIP39 immunoreactivity was observed in the fibers caudal to the knife cut, while it disappeared completely from the ipsilateral hypothalamus rostral to the transection.

Our tracer studies suggest that TIP39 neurons in the PIL project to the arcuate nucleus and the preoptic area in the hypothalamus and that neurons projecting to these brain regions are confined to the PIL within the posterior thalamus. The distribution of TIP39 neurons projecting to both hypothalamic sites were evenly distributed within the PIL. These results support that the PIL is a topographical unit although it does not correspond to an obvious cytoarchitectonically defined nucleus. In addition, the lack of retrograde labeling in other TIP39 cell groups suggests that TIP39 fibers in the arcuate nucleus and the preoptic area originate exclusively from the PIL.

Based on the data obtained by our injections of retrograde tracer into the PIL identified by the presence of TIP39 neurons, the PIL receives significant direct ascending projections as well as indirect projections via

the gracile and cuneate nuclei from the contralateral spinal cord. The direct projections were shown to arise from a wide range of thoracic and lumbar segments, mostly from neurons residing in deep layers of the dorsal horn of the spinal cord. Both of these afferent connections are candidates to convey suckling information from the nipples to the PIL.

2. Activation of TIP39 neurons in mother rats

Induction of TIP39 in the PIL and MPL of lactating mother rats was suggested on the basis of *in situ* hybridization histochemistry and confirmed by the independent technique of RT-PCR. The increased number of immunolabeled neurons observed in the PIL and MPL of lactating mothers suggests that the elevated level of mRNA expression of TIP39 is also reflected in an increased synthesis of TIP39 peptide.

On the basis of our *in situ* hybridization experiments we concluded that TIP39 mRNA levels were increased in the PIL on day 1 postpartum but not during pregnancy. TIP39 mRNA levels remained elevated throughout the lactation period but decreased to the basal level in response to the deprivation of pups.

It is particularly striking that TIP39 expression is induced only in the PIL, whereas the medial subparafascicular TIP39 cell group in the PVG continues to express a low level of TIP39 in lactating dams. Although TIP39 disappears from the PIL earlier than from the PVG during ontogeny, the adult levels are markedly reduced in both regions. Furthermore, brain areas that receive TIP39 axons exclusively from the PVG, including the lateral septal nucleus and the medial prefrontal cortex, also possess a high PTH2R level, suggesting that this TIP39 cell group may also be activated in response to some physiological stimuli.

Temporal pattern of TIP39 induction in the MPL is the same as in the PIL. In spite of this fact, these two TIP39 cell groups belong to two independent nuclei, which cytoarchitectonically and by their different afferent neuronal connections contrast strikingly with each other.

Fos, an immediate early gene expressed in activated cells, appears in the nuclei of TIP39 neurons of the PIL in response to suckling indicating an elevated activity of TIP39 neurons in lactating rat dams in this area. In this study, we investigated which stimulus activates TIP39 neurons in mother rats. We established that suckling has activated the TIP39 neurons in the PIL, but olfactory, visual and auditory inputs from pups without suckling did not.

3. Antagonism of the endogenous TIP39 suppresses suckling-induced prolactin release in mother rats

Suckling is the most potent known stimulus of the maternal increase in serum prolactin levels, however, the mechanisms are only partially understood. The experimental design of suckling-induced prolactin release is known to elevate plasma prolactin levels within minutes of pups' return to the mothers deprived of pups for 4 h, and plasma prolactin concentrations peak 30 min after the beginning of suckling. Saline injection into the lateral ventricle did not influence the prolactin level elicited by suckling, which matched the expected curve. Injection of the PTH2 receptor antagonist HYWY-TIP39, however, dose-dependently blocked the elevation of plasma prolactin levels. HYWY-TIP39 binds selectively to the PTH2 receptor, suggesting that HYWY-TIP39 injected into the lateral ventricle exerted its inhibitory action on the suckling-induced prolactin release via the PTH2 receptor. PTH2 receptor -expressing neurons are abundant in the

periventricular and arcuate nuclei of the hypothalamus. PTH2 receptors in these neurons are possible targets mediating the effect of HYWY-TIP39 on prolactin release. However, a direct effect of HYWY-TIP39 on dopaminergic neurons is not likely because the PTH2 receptor was not double labeled with tyrosine hydroxylase, and because close appositions between tyrosine hydroxylase neurons and fiber terminals projecting to the mediobasal hypothalamus from the PIL were not detected. Therefore, HYWY-TIP39 might influence dopaminergic neurons in the mediobasal hypothalamus via interneurons expressing the PTH2 receptor. Dynorphin-containing neurons in the arcuate nucleus are one of the candidates because they are innervated by axon terminals derived from the PIL, innervate tuberoinfundibular dopaminergic neurons, and may be responsible for the effects of opioid peptides on suckling-induced prolactin release by inhibiting tuberoinfundibular dopaminergic neurons.

We used a lentivirus to produce and constitutively secrete the selective PTH2 receptor antagonist HYWH-TIP39 from infected cells. Injection of this virus into the mediobasal hypothalamus reduced the basal prolactin levels in lactating mothers and markedly inhibited the suckling-induced elevation of plasma prolactin. Based on the effectiveness of the treatment, the secreted antagonist could diffuse some distance to reach PTH2 receptors and bind to them. PTH2 receptor-expressing neurons and PTH2 receptor-containing nerve terminals are absent from the pituitary but are abundant in the arcuate, paraventricular, and periventricular nuclei of the hypothalamus, which are therefore the likely sites of action of the antagonist secreted from the virus-infected cells in the mediobasal hypothalamus.

The reduced prolactin release in the presence of the PTH2 receptor antagonist suggests a physiological function of endogenous TIP39 in the

regulation of prolactin release. TIP39 fibers and fiber terminals are abundant in the mediobasal hypothalamus, with particularly high density in the arcuate, paraventricular and periventricular nuclei. These TIP39-containing nerve terminals are the likely source of endogenous TIP39 affecting prolactin release. Our findings provide strong evidence that these fibers originate from the PIL as anterograde tracer injected into the PIL demonstrated projections to the area while injection of retrograde tracer to the arcuate nucleus labeled TIP39 neurons in the PIL. An increased usage of TIP39 originated from the PIL is also supported by the elevated mRNA level of TIP39 in the PIL. The specific action of TIP39 during lactation is consistent with our findings that TIP39 mRNA level is not increased until the end of pregnancy and that it returns to the basal levels after the pups are removed from the mothers. Our finding on the role of the TIP39-PTH2 receptor system and its physiological significance in lactation is also consistent with the reduced body weight of foster pups reared by mice lacking the PTH2 receptor.

4. Antagonism of endogenous TIP39 reduces maternal motivation

The medial preoptic area has been shown to be critically important for maternal motivation through its projections to the nucleus accumbens and the ventral tegmental area. In the present study, we not only confirmed the role of the medial preoptic area in maternal motivation but also provided evidence for the involvement of the TIP39-PTH2 receptor system there. We demonstrated that fibers of TIP39 neurons projecting to the preoptic area from the PIL have a distribution similar to that of the neurons expressing Fos in response to pup exposure in areas that include the medial preoptic nucleus, the medial preoptic area, and the ventral subdivision of the bed

nucleus of the stria terminalis. This is a characteristic pattern often referred to as the medial preoptic area in which Fos-expressing neurons have been implicated in pup attachment. Most importantly, however, the presence of the PTH2 receptor antagonist reduced the number of dams demonstrating preference for the pup-associated cage in a place preference test and also the time the dams spent in the pup-associated cage. The increased time spent by the control virus injected dams in the pup-associated chamber as compared to the control cage indicates pup-seeking behavior. In contrast, the presence of the PTH2 receptor antagonist in the preoptic area eliminated the preference for the pup-associated chamber. Our finding that PTH2 receptor antagonist secretion in the preoptic area eliminated the preference for the pup-associated chamber suggests a role of the TIP39-PTH2 receptor system in associative learning, memory storage, or maternal motivation. However, the preoptic area has been shown to play a role in maternal motivation but not in memory formation and storage: lesions of the medial preoptic area reduced bar pressing for pups but not for food while inactivation of the medial preoptic area after memory formation eliminated pup-associated but did not affect cocaine-associated place preference. It is also important to note that preoptic injection of the virus expressing the PTH2 receptor antagonist did not affect plasma prolactin levels. Therefore, an indirect mechanism of action *via* prolactin can be excluded.

We compared the behavior of mother rats that received preoptic area virus injections (PTH2 receptor antagonist expressing virus-injected mothers, control virus-injected mothers) using a conditioned place preference test. Control virus-injected mothers demonstrate preference towards a cage visually similar to the one they had been previously housed with their litter. In contrast, injection of a virus continuously expressing a peptide antagonist of the PTH2 receptor does not show preference for the

familiar cage. The PTH2 receptor antagonist expressing animals spent less time in the pup-associated cage than the control mothers. A time index for preference $100 * (\text{time spent in the pup associated cage} - \text{time spent in the control cage}) / (\text{time spent in the pup associated cage} + \text{time spent in the control cage})$ was also significantly higher in mothers injected with the control virus than those injected with the PTH2 receptor antagonist expressing virus.

The reduced pup-associated place preference following the expression of the PTH2 receptor antagonist suggests a physiological role of endogenous TIP39 in the control of maternal motivation. TIP39-containing terminals, surrounding Fos-expressing neurons in the medial preoptic area, are potential endogenous source of TIP39 influencing maternal motivation. Previously, we also demonstrated that PTH2 receptors are present in the preoptic area and their distribution is very similar to that of TIP39 terminals in the area. The results of our tracer studies provide strong evidence that these TIP39 fibers arise from the PIL as the injection of an anterograde tracer into the PIL revealed projections to the area while injection of a retrograde tracer into the medial preoptic area labeled TIP39 neurons in the PIL. However, the timing of the expression of TIP39 mRNA on the first postpartum day suggests that TIP39 has a role in the maintenance of maternal motivation rather than in its induction. Suckling and pup contact may, in turn, be responsible for the maintenance of maternal motivation and behavior in dams.

TIP39 neurons are ideally positioned in the posterior thalamus to convey suckling information towards forebrain maternal centers. They relay sensory signals from pups to the medial preoptic area, which affect other motivational centers and turn the mother's motivational stance to her pups.

V. CONCLUSIONS

Our most important novel findings are as follows:

1. We established that the posterior intralaminar complex of the thalamus can be divided into two subdivisions on the basis of existence of neuronal markers. TIP39 neurons located in the medial subdivision were surrounded by galanin fibers. While CGRP neurons can be found lateral to TIP39 neurons. We reported that calbindin but not calretinin or parvalbumin co-expresses with TIP39 in the PIL. Thereby we established a marker of TIP39 neurons in the PIL.

2. By means of tract tracer studies, transections and Fos activation techniques, we demonstrated that TIP39 neurons in the PIL project to hypothalamic nuclei, which participate in the regulation of maternal adaptations. Neurons in the PIL receive indirect ascending information from the nucleus gracilis and cuneatus, the relay nuclei of fasciculus dorsalis and lemniscus medialis. PIL also receive direct ascending information from the spinal cord, probably through collaterals of tractus spinothalamicus. Suckling activates PIL neurons, since Fos expression was significantly higher in nursing mothers, than in the case of mother-pup coexistence without close contact. In earlier studies, Fos-expressing neurons were described in deep layers of thoracic and lumbar spinal cord in suckled dams, exactly where we noticed retrograde-labeled neurons after injection of CTb to the PIL supporting that TIP39 neurons convey suckling information from spinal cord towards the hypothalamus.

3. Using real-time RT-PCR, *in situ* hybridization histochemistry, and immunohistochemistry, we found that TIP39 mRNA and peptide levels are markedly elevated in the PIL and the MPL of lactating dams compared to the PIL and MPL of virgin control and postpartum female rats separated from their pups. These results imply that TIP39 may have a role in the regulation of maternal adaptations. The position of the MPL immediately next to the nuclei of the lateral lemniscus and its bilateral anatomical connections with auditory brain regions suggests some auditory functions of paralemniscal TIP39 neurons. In rats, paralemniscal TIP39 neurons were specifically activated by high-intensity noise, so specific auditory inputs could play a role in the activation of TIP39 neurons in mothers. We hypothesize that paralemniscal TIP39 neurons may be activated in mother rats by ultrasonic vocalization of pups. In turn, paralemniscal TIP39 neurons could mediate pup ultrasonic vocalization towards higher brain centers of their mothers thereby contributing to central maternal adaptations.

4. We provided evidence that TIP39 has an effect on prolactin release through thalamo-arcuate projection and on maternal motivation through thalamo-preoptic projection. We revealed using two different methods that PTH2 receptor antagonism blocks the suckling induced prolactin release. We first proved that TIP39 released from mediobasal hypothalamus plays a role in the regulation of prolactin secretion. We also demonstrated that TIP39 released from the medial preoptic area contributes to formation and maintenance of maternal motivation. The identified anatomical pathways and neurochemical substrates represent breakthroughs in our understanding of the mechanisms how nursing affects the maternal brain. Because the

TIP39-PTH2 receptor system is neuroanatomically similar in humans and rodents, the results may have implications relevant to human breastfeeding.

VI. LIST OF PUBLICATIONS

1. Publications related to the thesis

1. **Cservenák M**, Bodnár I, Usdin TB, Palkovits M, Nagy GM, Dobolyi A. (2010) Tuberoinfundibular peptide of 39 residues is activated during lactation and participates in the suckling-induced prolactin release in rat. *Endocrinology*, 151: 5830-5840. **IF: 4,993**

2. Varga T, Mogyoródi B, Bagó AG, **Cservenák M**, Domokos D, Renner E, Gallatz K, Usdin TB, Palkovits M, Dobolyi A. (2012) Paralemniscal TIP39 is induced in rat dams and may participate in maternal functions. *Brain Struct Funct*, 217: 323-335. **IF: 7,837**

2. Abstracts related to the thesis

1. **Cservenák M**, Usdin TB, Palkovits M, Dobolyi A. (2009) Activation of TIP39 neurons in the lateral subparafascicular area of mother rats. 12th Meeting of the Hungarian Neuroscience Society (MITT), Budapest. *Frontiers in Systems Neuroscience*. Conference Abstract, doi: 10.3389/conf.neuro.01.2009.04.007.

2. **Cservenák M**, Usdin TB, Palkovits M, Dobolyi A. (2009) TIP39-neuropeptidet szintetizáló idegsejtek a posterior intralamináris talamikus magokban: aktiválódás szoptatós anya patkányokban. A Magyar Anatómus Társaság 15. Kongresszusa, Budapest.

3. **Cservenák M**, Bodnár I, Usdin TB, Palkovits M, Nagy GM, Dobolyi A. (2010) Intracerebroventricular injection of a parathyroid hormone 2 receptor antagonist abolishes suckling induced prolactin release. International IBRO Workshop, Pécs. *Frontiers in Systems Neuroscience*. Conference Abstract: IBRO International Workshop 2010. doi: 10.3389/conf.fnins.2010.10.00081.

4. **Cservenák M**, Bodnár I, Usdin TB, Palkovits M, Nagy GM, Dobolyi A. (2011) Tuberoinfundibular peptide of 39 residues is activated during lactation and participates in the suckling-induced prolactin release in rat. 13th Conference of the Hungarian Neuroscience Society (MITT), Budapest. *Frontiers in Systems Neuroscience*. Conference Abstract, doi: 10.3389/conf.fnins.2011.84.00108.

5. **Cservenák M**, Bodnár I, Usdin TB, Palkovits M, Nagy GM, Dobolyi A. (2011) A new component in the regulation of prolactin release in lactation: The TIP39-PTH2 receptor system. The Joint Conference (FAMÉ 2011) of the LXXVth Meeting of the Hungarian Physiological Society, XVIth Meeting of the Hungarian Society of Anatomists, Experimental Section of the Hungarian Society for Experimental and Clinical Pharmacology and Hungarian Society for Microcirculation and Vascular Biology, Pécs. *Acta Physiologica* 2011; Volume 202, Supplement 684: P14.

6. Dobolyi A, **Cservenak M**, Usdin TB, Palkovits M. (2011) Maternally activated posterior thalamic neurons possibly convey the suckling information towards hypothalamic centers. 93rd Annual Meeting & Expo of

the Endocrine Society (ENDO), Boston, USA, Endocrine Reviews, 32: P3-235.

7. **Cservenák M**, Bodnár I, Nagy GM, Usdin TB, Palkovits M, Dobolyi A. (2012) Posterior thalamic TIP39 neurons project to the medial hypothalamus and regulate prolactin secretion in mothers. IBRO International Workshop, Szeged. Clinical Neuroscience 2012; Volume 65, Supplement 1: P14.

3. Publication not related to the thesis

1. Szabó ER, **Cservenák M**, Dobolyi A. (2012) Amylin is a novel neuropeptide with potential maternal functions in the rat. FASEB J, 26: 272-281. **IF: 5,704**

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