A KUTATÁS EREDMÉNYEIT ÖSSZEFOGLALÓ RÉSZLETES JELENTÉS (REPORT ON THE RESULTS OF THE RESEARCH PROJECT)

Novel peptiderg mechanisms in central maternal adaptations

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Tuberoinfundibular peptide of 39 residues (TIP39) is a neuropeptide we cloned before the project and determined its distribution in the brain. In the framework of the application, mRNA levels were measured in all three brain sites of its expression, using in situ hybridization histochemistry and RT-PCR. TIP39 mRNA levels increased about 6 times in the posterior intralaminar complex of the thalamus (PIL), did not change in the subparafascicular area of the thalamus, and increased 4 times in the paralemniscal area situated between the pontine reticular formation and the lateral lemniscus in the pontomesencephalic tegmentum in lactating mothers as opposed to nulliparous females and mothers deprived of pups using real-time RT-PCR (1).

In situ hybridization histochemistry and immunolabeling in the paralemniscal area demonstrated that the induction of TIP39 in mothers takes place within the medial paralemniscal nucleus (MPL), a cytoarchitectonically distinct part of the paralemniscal area, and that the increase in TIP39 mRNA levels translates into elevated peptide levels dams. The paralemniscal area has been implicated in maternal control as well as in pain perception. To establish the function of induced TIP39, we investigated the activation of TIP39 neurons in response to pup exposure as maternal, and formalin injection as noxious stimulus. Both stimuli elicited *c-fos* expression in the paralemniscal area. Subsequent double labeling demonstrated that 95% of neurons expressing Fos in response to pup exposure also contained TIP39 immunoreactivity and 91% of TIP39 neurons showed *c-fos* activation by pup exposure. In contrast, formalin-induced Fos did not co-localize with TIP39. Instead, most formalin-activated neurons are situated medial to the TIP39 cell group. Our data indicate that paralemniscal neurons may be involved in the processing of maternal and nociceptive information. However, two different groups of paralemniscal neurons participate in the two functions. In particular, TIP39 neurons may participate in the control of maternal functions (2).

TIP39 synthesizing neurons showing elevated TIP39 levels in mother rats in the PIL was further investigated. An experiment was performed to describe the activation of TIP39 neurons in the PIL in response to suckling the litter. When pups were returned to their mothers after a 20h separation, the dams all began care for them and nursed them immediately. Following pup return, c-Fos-expressing neurons appeared in a number of regions in the dams' brains including the PIL (and also the lateral septal nucleus, anteroventral periventricular nucleus, medial preoptic nucleus, medial preoptic area, the ventral subdivision of the bed nucleus of the stria terminalis, some parts of the periaqueductal gray, and the medial paralemniscal nucleus). While c-Fos-ir neurons were evenly distributed within the PIL, none appeared in adjacent regions, except in the peripeduncular area lateral to

the PIL. In the PIL section with the largest number of labeled cells, the number of Fos-ir neurons changed significantly with pup exposure. In suckling dams, it was significantly greater than in dams following pup exposure without any physical contact. TIP39 fibers were present in the hypothalamic arcuate nucleus, which regulates lactation. Therefore, we addressed the function of posterior intralaminar TIP39 neurons in lactation. To evaluate a potential causal relationship between TIP39 signaling and prolactin secretion from the pituitary in response to suckling we infected cells in the mediobasal hypothalamus near the arcuate nucleus with a virus encoding the parathyroid hormone 2 receptor (PTH2-receptor, the receptor of TIP39) antagonist HYWH-TIP39. Basal plasma prolactin levels in the mother rats expressing HYWH-TIP39 were significantly lower than that in the control dams. After 4h of separation from their pups, levels of plasma prolactin in the two groups of mothers had fallen to similar levels. When pups were returned after the 4h separation period, attachment and suckling began within less than 5 min for all animals. The control virus and the PTH2 receptor antagonist-expressing virus injected animals had a significantly different prolactin response. The increase in plasma prolactin was significantly less in the antagonist expressing dams. In contrast, virus injected into the preoptic area did not significantly change either the plasma prolactin level (3).

At the preoptic level, the anteroventral periventricular nucleus, the medial preoptic nucleus, the medial preoptic area, and the ventral subdivision of the bed nucleus of the stria terminalis all contained a high density of Fos-expressing neurons following suckling. The distribution pattern of Fos-expressing neurons was very similar to the distribution patterns of TIP39 labeled fibers and terminals observed in the area. TIP39 containing fibers appeared to closely appose Fos-expressing neurons in all regions of the preoptic area that contain Fos-expressing neurons following suckling. Since the preoptic area plays a role in the control of maternal motivation, the role of TIP39 in maternal motivation was addressed by antagonizing its receptor. Preoptic area virus injections resulted in a number of infected cells similar to the mediobasal hypothalamic injections. The behavior of mother rats that received virus injections into the preoptic area was analyzed using a place preference test. Defining a preferred cage as one that animals spend at least 20% more time in than the non-preferred cage, 10 out of 11 control dams preferred the pup associated cage. Dams injected in the preoptic area with the PTH2 receptor antagonist expressing virus had a significantly different cage preference as only 5 out of 13 rats preferred the pup-associated cage, while 4 rats showed preference for the control cage. The amount of time spent in the different compartments was also significantly different in the 2 groups suggesting the involvement of the TIP39-PTH2 receptor system in the control of maternal motivation (3, 4).

Amylin is a 37 amino acid peptide, which we previously found in the preoptic area of mother rats in a microarray study. The time course and mechanisms of amylin induction, as well as its functions, were investigated in the rat. Examining the time course of amylin induction, we found that amylin is not expressed in the brain before and during pregnancy but its significant increase was observed in rats and mice immediately after parturition in the preoptic area, a region whose lesion abolishes maternal behaviors. Ovariectomy had no effect on the activation of amylin neurons suggesting sexual steroid independent mechanisms.

Amylin was expressed in the medial preoptic nucleus, parts of the medial preoptic area and the bed nucleus of the stria terminalis during the postpartum period as long as mothers were not separated from pups. Amylin expression was also induced in maternally behaving (sensitized) but not in non-sensitized ovariectomized nulliparous females using *in situ* hybridization histochemistry suggesting a limited role of sexual steroids in amylin expression. Quantitative RT-PCR demonstrated elevated amylin mRNA levels in maternally sensitized nulliparous female rats. Immunohistochemistry verified the increased amylin expression in maternally behaving rats and demonstrated the same expression pattern of amylin as *in situ* hybridization histochemistry. In a subsequent experiment, mothers, separated from their pups for 22 hours, expressed c-fos in their preoptic area after returning the pups. The distribution pattern of activated and amylin-expressing neurons was similar. Double labeling of Fos and amylin using double fluorescent immunocytochemistry or a combination of Fos immunolabeling with *in situ* hybridization histochemistry for amylin revealed that 92-95% of amylin neurons were activated by pup exposure. The results implicate amylin in the control of maternal adaptations possibly exerting its actions *via* amylin receptors present in brain regions to which preoptic neurons project (5).

As a continuation of our studies to understand how ascending pathways affect lactation and maternal motivation in the postpartum period, the anterograde tracer biotinylated dextran amine was injected into a cell group expressing TIP39 in the PIL. It was revealed that TIP39 neurons in the PIL project to established maternal centers, including the preoptic area and the arcuate nucleus. In particular, the projection of PIL TIP39 neurons includes innervation of amylin neurons in the preoptic area as shown by double labeling techniques. The interaction between the 2 peptides was also suggested by an experiments demonstrating that the induction of amylin in the preoptic area of lactating dams depends on TIP39-containing neurons in the PIL because amylin levels were markedly reduced in TIP39 knock-out mice. To establish the effect of the lack of amylin on behavior, amylin knock-out mice were used. First, we had to evaluate these behaviors in wild-type mice as the species differs substantially from the better characterized rats. In our study, behavioral changes in virgin female, maternally behaving sensitized virgin female, and primiparous mother mice were investigated using three behavioral tests measuring active maternal behavior, maternal motivation and depression-like behavior. Maternal responsiveness can actually develop in response to pup exposure even in the absence of hormonal stimulation, in a process termed sensitization. In the pup retrieval test measuring active maternal behavior, latency to retrieve pups was not significantly different between the 3 female groups. In the second experiment, which tested maternal motivation, the conditioned place preference (CPP) test was used. Mother and maternally sensitized female mice spent significantly more time in the pup-associated cage than in the control cage, while the virgin female mice significantly preferred the control cage during the 1 h test period. In the third experiment, the forced swim test (FST) was used to examine depression-like behavior. The time spent with active (swimming and struggling) and passive (floating) behavior was measured for 6 min. The active behavior in FST showed significant differences among the maternal status: mother mice spent more time with active behavior than virgin and sensitized female mice. The results indicate that control mice have more pronounced spontaneous maternal behavior than the much better described

rats. Still, behavioral alterations can be detected in mice, too, when they become mothers in particular regarding maternal motivation and depression-like behavior, similar to rats. In addition, a maternal sensitization procedure can have measurable effects in mice even with a substantially reduced sensitization procedure as compared to rats. Interestingly, however, the anti-depression-like behavior characteristic of mother mice is not present in the sensitized animals (6, 7).

Selective ablation of preoptic galanin neurons resulted in cessation of the parental behavior and emergence of pup-directed aggression as recently reported by others. we identified a group of galanin neurons in the anterior commissural nucleus (ACN), and a distinct group in the medial preoptic area (MPA). Galanin neurons in ACN but not the MPA co-expressed oxytocin. We used immunodetection of phosphorylated STAT5 (pSTAT5), involved in prolactin receptor signal transduction, to evaluate the effects of suckling-induced prolactin release and found that 76 % of galanin cells in ACN, but only 12% in MPA were prolactin responsive. Nerve terminals containing TIP39, a neuropeptide that mediates effects of suckling on maternal motivation, were abundant around galanin neurons in both preoptic regions. In the ACN and MPA, 89 and 82 % of galanin neurons received close somatic appositions, with an average of 2.9 and 2.6 per cell, respectively. We observed perisomatic innervation of galanin neurons using correlated light and electron microscopy. The connection was excitatory based on the glutamate content of TIP39 terminals demonstrated by post-embedding immunogold electron microscopy. Injection of the anterograde tracer biotinylated dextran amine into the TIP39-expressing PIL demonstrated that preoptic TIP39 fibers originate in the PIL, which is activated by suckling. Thus, galanin neurons in the preoptic area of mother rats are innervated by an excitatory neuronal pathway that conveys suckling-related information. In turn, they can be topographically and neurochemically divided into two distinct cell groups, of which only one is affected by prolactin (8).

Oxytocin is released from neurons in the paraventricular hypothalamic nucleus (PVN) in mothers upon suckling and during adult social interactions. However, neuronal pathways that activate oxytocin neurons in social contexts are not established yet. Innervation of oxytocin neurons by TIP39 neurons was examined by double labeling in combination with electron microscopy and retrograde tract-tracing. Potential classical neurotransmitters in TIP39 neurons were investigated by in situ hybridization histochemistry. Neurons activated after encounter with a familiar conspecific female in familiar environment were mapped with the c-fos technique. PVN and the supraoptic nucleus oxytocin neurons were closely apposed by an average of 2.0 and 0.4 TIP39 terminals, respectively. Asymmetric (presumed excitatory) synapses were found between TIP39 terminals and cell bodies of oxytocin neurons. In lactating rats, PIL TIP39 neurons were retrogradely labeled from the PVN. TIP39 neurons expressed vesicular glutamate transporter 2 but not glutamic acid decarboxylase 67. PIL contained a markedly increased number of c-Fos-positive neurons in response to social encounter with a familiar conspecific female. Furthermore, the PIL received ascending input from the spinal cord and the inferior colliculus. Thus, TIP39 neurons in the PIL may receive sensory input in response to social interactions and project to the PVN to innervate and excite

oxytocin neurons suggesting that the PIL-PVN projection contributes to the activation of oxytocin neurons in social contexts (9).

The interaction of TIP39 and amylin with other neuropeptides, nucleosides, notch, and transforming growth factor beta protein receptors was also investigated (10-15). It was established that these neuroactive molecules are present in brain areas containing TIP39 fibers (16-18,25). Thus, TIP39 could influence their activity potentially contributing to their different functions in the brain (19-22).

The highest center of maternal responsiveness lies within the medial prefrontal cortex, where TIP39 fibers also terminate. Therefore, we dissected this brain region to investigate proteomic changes in rat dams. The control group consisted of mothers, which were deprived of their pups immediately after the parturition. 2-DIGE minimal dye technique combined with LC-MS/MS was used to separate proteins and determine protein level differences. We identified 32 different significant protein changes in the medial prefrontal cortex. The altered proteins participate in synaptic transport and plasticity, neuron development, oxidative stress and apoptosis, cytoskeleton organization, ion transport, protein and glucose metabolism, cell cycle and differentiation, based on the classification. Alpha-crystallin B chain (Cryab), a small heat-shock protein showed the greatest protein level changes. Its protein level increase was validated with WB technique. The location of cells expressing Cryab was described with immunohistochemistry. The density of Cryab-immunopositive cells was higher in the maternal than in the non-maternal prefrontal cortex. The labeled cells had relatively widespread distribution but were most abundant in layer IV of the prelimbic and infralimbic cortices. Double labeling revealed that Cryab immunoreactivity was present in parvalbumin- but not in calbindin-positive neurons suggesting that some GABAergic interneurons are involved in the brain maternal adaptation and that Cryab plays a role in this process (23).

Finally, postmortem human brain tissue samples were obtained from the Human Brain Tissue Bank. The rate of degradation of the mRNA was determined in the human tissue samples. Subsequently, immunolabeling and in situ hybridization histochemistry was developed in human samples. These approaches led to the discovery of localized expression of succinyl CoA ligase subunit A2 in the neurons as this enzyme was not found in any glial cell type in the human brain (24).

The results of the project were published in an MTA Doctoral dissertation of the principal investigator, 24 research articles with NKFIH/OTKA support (7 D1, additional 9 Q1 papers). The principal investigator was corresponding senior author in all D1 and 5 Q1 papers.

Publications of the Results of the Project

1. Dobolyi, A. (2014) Two novel neuropeptides with maternal functions. *Doctoral Dissertation of the Hungarian Academy of Sciences*

2. Varga, T., Mogyorodi, B., Bago, A. G., Cservenak, M., Domokos, D., Renner, E., Gallatz, K., Usdin, T. B., Palkovits, M., and Dobolyi, A. (2012) Paralemniscal TIP39 is induced in rat dams and may participate in maternal functions. *Brain Struct Funct* **217**, 323-335
   Folyóirat szakterülete: Histology helyzete: D1

3. Cservenak, M., Szabo, E. R., Bodnar, I., Leko, A., Palkovits, M., Nagy, G. M., Usdin, T. B., and Dobolyi, A. (2013) Thalamic neuropeptide mediating the effects of nursing on lactation and maternal motivation. *Psychoneuroendocrinology* **38**, 3070-3084
   Folyóirat szakterülete: Endocrinology helyzete: D1

4. Dobolyi, A., Dimitrov, E., Palkovits, M., and Usdin, T. B. (2012) The neuroendocrine functions of the parathyroid hormone 2 receptor. *Front Endocrinol (Lausanne)* **3**, 121
   Folyóirat szakterülete: Endocrinology, Diabetes and Metabolism helyzete: Q3

5. Szabo, E. R., Cservenak, M., and Dobolyi, A. (2012) Amylin is a novel neuropeptide with potential maternal functions in the rat. *FASEB J* **26**, 272-281
   Folyóirat szakterülete: Medicine (miscellaneous) helyzete: D1

6. Dobolyi, A., Grattan, D. R., and Stolzenberg, D. S. (2014) Preoptic inputs and mechanisms that regulate maternal responsiveness. *J Neuroendocrinol* **26**, 627-640
   Folyóirat szakterülete: Endocrinology helyzete: Q1

7. Szabo, É. R., Cservenak, M., Lutz, T. A., Gévai, L., Endrényi, M., Simon, L., and Dobolyi, A. (2015) Behavioural changes in mothers and maternally sensitised female mice. *Behaviour* **152**, 1801-1819
   Folyóirat szakterülete: Animal Science and Zoology helyzete: Q1

8. Cservenak, M., Kis, V., Keller, D., Dimen, D., Menyhart, L., Olah, S., Szabo, E. R., Barna, J., Renner, E., Usdin, T. B., and Dobolyi, A. (2016) Maternally involved galanin neurons in the preoptic area of the rat. *Brain Struct Funct*
   Folyóirat szakterülete: Histology helyzete: D1

9. Cservenak, M., Keller, D., Kis, V., Fazekas, E. A., Ollos, H., Leko, A., Szabo, E. R., Renner, E., Usdin, T. B., Palkovits, M., and Dobolyi, A. (2016) A thalamo-hypothalamic pathway that activates oxytocin neurons in social contexts in female rats. *Endocrinology*, en20161645
   Folyóirat szakterülete: Endocrinology helyzete: D1

10. Kovacs, Z., Kekesi, K. A., Juhasz, G., Barna, J., Heja, L., Lakatos, R., and Dobolyi, A. (2015) Non-adenosine nucleoside inosine, guanosine and uridine as promising antiepileptic drugs: a summary of current literature. *Mini Rev Med Chem* **14**, 1033-1042

Folyóirat szakterülete: Medicine (miscellaneous) helyzete: Q2

11. Nardai, S., Dobolyi, A., Pal, G., Skopal, J., Pinter, N., Lakatos, K., Merkely, B., and Nagy, Z. (2015) Selegiline promotes NOTCH-JAGGED signaling in astrocytes of the peri-infarct region and improves the functional integrity of the neurovascular unit in a rat model of focal ischemia. *Restor Neurol Neurosci* **33**, 1-14
Folyóirat szakterülete: Neurology (clinical) helyzete: Q1

12. Kovacs, Z., Dobolyi, A., Kekesi, K. A., and Juhasz, G. (2013) 5'-nucleotidases, nucleosides and their distribution in the brain: pathological and therapeutic implications. *Curr Med Chem* **20**, 4217-4240
Folyóirat szakterülete: Pharmacology helyzete: Q1

13. Kovacs, Z., Slezia, A., Bali, Z. K., Kovacs, P., Dobolyi, A., Szikra, T., Hernadi, I., and Juhasz, G. (2013) Uridine modulates neuronal activity and inhibits spike-wave discharges of absence epileptic Long Evans and Wistar Albino Glaxo/Rijswijk rats. *Brain Res Bull* **97**, 16-23
Folyóirat szakterülete: Neuroscience (miscellaneous) helyzete: Q2

14. Pal, G., Vincze, C., Renner, E., Wappler, E. A., Nagy, Z., Lovas, G., and Dobolyi, A. (2012) Time course, distribution and cell types of induction of transforming growth factor betas following middle cerebral artery occlusion in the rat brain. *PLoS One* **7**, e46731
Folyóirat szakterülete: Medicine (miscellaneous) helyzete: D1

15. Dobolyi, A., Vincze, C., Pal, G., and Lovas, G. (2012) The neuroprotective functions of transforming growth factor beta proteins. *Int J Mol Sci* **13**, 8219-8258
Folyóirat szakterülete: Molecular Biology helyzete: Q3

16. Kovacs, Z., Dobolyi, A., Juhasz, G., and Kekesi, K. A. (2014) Lipopolysaccharide induced increase in seizure activity in two animal models of absence epilepsy WAG/Rij and GAERS rats and Long Evans rats. *Brain Res Bull* **104**, 7-18
Folyóirat szakterülete: Neuroscience (miscellaneous) helyzete: Q2

17. Dobolyi, A., Kekesi, K. A., Juhasz, G., Szekely, A. D., Lovas, G., and Kovacs, Z. (2014) Receptors of peptides as therapeutic targets in epilepsy research. *Curr Med Chem* **21**, 764-787
Folyóirat szakterülete: Pharmacology helyzete: Q1

18. Kovacs, Z., Kekesi, K. A., Juhasz, G., and Dobolyi, A. (2014) The antiepileptic potential of nucleosides. *Curr Med Chem* **21**, 788-821
Folyóirat szakterülete: Pharmacology helyzete: Q1

19. Kovacs, Z., Kekesi, K. A., Juhasz, G., and Dobolyi, A. (2015) Modulatory effects of inosine, guanosine and uridine on lipopolysaccharide-evoked increase in spike-wave discharge activity in Wistar Albino Glaxo/Rijswijk rats. *Brain Res Bull* **118**, 46-57
Folyóirat szakterülete: Neuroscience (miscellaneous) helyzete: Q2

20. Kovacs, Z., Kekesi, K. A., Dobolyi, A., Lakatos, R., and Juhasz, G. (2015) Absence epileptic activity changing effects of non-adenosine nucleoside inosine, guanosine and uridine in Wistar Albino Glaxo Rijswijk rats. *Neuroscience* **300**, 593-608
Folyóirat szakterülete: Neuroscience (miscellaneous) helyzete: Q1

21. Lakatos, R. K., Dobolyi, A., Todorov, M. I., Kekesi, K. A., Juhasz, G., Aleksza, M., and Kovacs, Z. (2016) Guanosine may increase absence epileptic activity by means of A2A adenosine receptors in Wistar Albino Glaxo Rijswijk rats. *Brain Res Bull* **124**, 172-181
Folyóirat szakterülete: Neuroscience (miscellaneous) helyzete: Q2

22. Nardai, S., Dobolyi, A., Skopal, J., Lakatos, K., Merkely, B., and Nagy, Z. (2016) Delayed Gelatinase Inhibition Induces Reticulon 4 Receptor Expression in the Peri-Infarct Cortex. *J Neuropathol Exp Neurol* **75**, 379-385
Folyóirat szakterülete: Neurology helyzete: Q1

23. Volgyi, K., Udvari, E. B., Szabo, E. R., Gyorffy, B. A., Hunyadi-Gulyas, E., Medzihradszky, K., Juhasz, G., Kekesi, K. A., and Dobolyi, A. (2016) Maternal alterations in the proteome of the medial prefrontal cortex in rat. *J Proteomics*
Folyóirat szakterülete: Biochemistry helyzete: Q1

24. Dobolyi, A., Bago, A. G., Gal, A., Molnar, M. J., Palkovits, M., Adam-Vizi, V., and Chinopoulos, C. (2015) Localization of SUCLA2 and SUCLG2 subunits of succinyl CoA ligase within the cerebral cortex suggests the absence of matrix substrate-level phosphorylation in glial cells of the human brain. *J Bioenerg Biomembr* **47**, 33-41
Folyóirat szakterülete: Physiology helyzete: Q2

25. Pál, G., Lovas G., Dobolyi, A. (2014) Induction of transforming growth factor beta receptors following focal ischemia in the rat brain. *PLoS One* 9, e106544
Folyóirat szakterülete: Medicine (miscellaneous) helyzete: D1

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