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The Function of Transforming Growth Factor beta Proteins in the Central Nervous System

Book chapters with the support of OTKA NNF2 85612

Dobolyi A. (2012) Transforming growth factor beta in the central nervous system In: Carlos M. Contreras, Editor, *Neuroscience – Dealing with Frontiers*, In Tech, 2012, Chapter 6, Pages 129-148. ISBN 978-953-51-0207-6.

Research articles with the support of OTKA NNF2 85612

1. Pál G., Vincze C., Renner É., Wappler E.A., Nagy Z., Lovas G., 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(10):e46731. doi: 10.1371/journal.pone.0046731.
   Impact factor: 4.092

2. Szabó E.R., Cservenák M., Dobolyi A. (2012) Amylin is a novel neuropeptide with potential maternal functions. *FASEB J*. 26, 272-281.
   Impact factor: 5.712

3. Varga T., Mogyoródi B., Bagó A.G., Cservenák M., Domokos D., Renner E., Gallatz K., Usdin T.B., Palkovits M., Dobolyi A. (2012) Paralemniscal TIP39 is induced in rat dams and may participate in maternal functions. *Brain Struct. Funct.* 217, 323–335.
   Impact factor: 5.628

4. Dobolyi A., Vincze C., Pál G., Lovas G. (2012) The neuroprotective functions of transforming growth factor beta proteins. *Int. J. Mol. Sci.* 13, 8219-8258.
   Impact factor: 2.598

5. Dobolyi A. (2011) Novel potential regulators of maternal adaptations during lactation: tuberoinfundibular peptide 39 and amylin. *J. Neuroendocrinol*. 23, 1002-1008.
   Impact factor: 3.138

6. Dobolyi A., Kovács Z., Juhasz G., Kardos J. (2011) Uridine function in the central nervous system. *Curr. Top. Med. Chem*. 11, 1058-67.
   Impact factor: 4.174

Conference abstracts with the support of OTKA NNF2 85612

1 Cservenák M., Bodnár I., Nagy G.M., Usdin T.B., Palkovits M., Dobolyi A. (2012) TIP39 neurons in the posterior thalamus are relay stations in the ascending pathway of suckling reflexes in mother rat., 8th Forum of European Neuroscience, Barcelona, Spain, FENS Abstract Vol, 4781.

2 Szabo E.R., Cservenak M., Domokos D., Dobolyi A. (2012) Identification of amylin as a novel neuropeptide in the brain of mother rats. XXI International Semmelweis Symposium, Budapest, 2012. Orvosképzés 87(2):327.

3 Pal G., Vincze C., Renner E., Wappler A.E., Nagy Z., Lovas G., Dobolyi A. (2012) Time course, distribution and cell types of induction of transforming growth factor betas in a rat model of ischemic stroke. XXI International Semmelweis Symposium, Budapest, 2012. Orvosképzés 87(2):315.

4 Cservenak M., Bodnar I., Nagy G.M., Usdin T.B., Palkovits M., Dobolyi A. (2012) Posterior thalamic TIP39 neurons project to the medial hypothalamus and regulate prolactin secretion in mothers. International IBRO Workshop, Szeged 2012. Clin. Neurosci./Ideggyogy. Sz. 65(S1):14.

5 Dobolyi A. (2012) Brain circuitry of maternal adaptations. International IBRO Workshop, Szeged 2012. Clin. Neurosci./Ideggyogy. Sz. 65(S1):16. Oral presentation.

6 Pal G., Vincze C., Renner E., Lovas G., Dobolyi A. (2012) Differential expression pattern of TGFb 1 and 2 in neural and glial cells following MCAO in rat brain. International IBRO Workshop, Szeged 2012. Clin. Neurosci./Ideggyogy. Sz. 65(S1):52.

7 Szabo E.R., Cservenak M., Dobolyi A. (2012) Amylin is a novel neuropeptide activated in the brain of mother rats. International IBRO Workshop, Szeged 2012. Clin. Neurosci./Ideggyogy. Sz. 65(S1):62.

8 Vincze C., Pal G., Wappler E.A., Nagy Z., Lovas G., Dobolyi A. (2012) The time course of TGFb 1, 2 and 3 expression following focal brain ischemia. International IBRO Workshop, Szeged 2012. Clin. Neurosci./Ideggyogy. Sz. 65(S1):75.

9 Dobolyi A., Vincze C., Pál G., Wappler A.E., Nagy Z., Lovas G. (2012) A transzformáló növekedési faktor beta 1, 2, és 3 proteinek indukciójának térbeli és időbeli lefutása fokális agyi ischemiát követően. A Magyar Élettani Társaság, a Magyar Anatómusok Társasága, a Magyar Biofizikai Társaság és a Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság Kongresszusa, Debrecen, Abstract No. 40.

10 Szabó É.R., Cservenák M., Dobolyi A. (2012) Egy új, anyapatkányokban aktiválódó neuropeptid, az amylin azonosítása. A Magyar Élettani Társaság, a Magyar Anatómusok Társasága, a Magyar Biofizikai Társaság és a Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság Kongresszusa. Debrecen, Abstract No. 48.

11 Cservenák M., Bodnár I., Nagy G.M., Usdin T.B., Palkovits M., Dobolyi A. (2012) Egy új neuromodulátor rendszer a szopási inger által kiváltott prolaktin felszabadulás szabályozásában. A Magyar Élettani Társaság, a Magyar Anatómusok Társasága, a Magyar Biofizikai Társaság és a Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság Kongresszusa, Debrecen, Abstract No. 100.

12 Pál G., Vincze C., Lovas G., Renner É., Dobolyi A. (2012) Transzformáló növekedési faktor-béta fehérjéket expresszáló sejtek típusának azonosítása fokális ischémiát követően patkányagyban. A Magyar Élettani Társaság, a Magyar Anatómusok Társasága, a Magyar Biofizikai Társaság és a Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság Kongresszusa, Debrecen, Abstract No. 118.

13 Cservenak M., Bodnár I., Usdin T. B., Palkovits M., Nagy G. M., Dobolyi A. (2011) A new player in the regulation of suckling-induced prolactin release in rat. 8th IBRO World Congress of Neuroscience, Florence, Italy, C151.

14 Szabó É. R., Tombácz D., Boldogkői Z., Palkovits M., Dobolyi A. (2011) Identification of injection sites for recombinant pseudorabies virus strains injected into the auditory cortex as retrograde tracers. 8th IBRO World Congress of Neuroscience, Florence, Italy, B244.

15 Dobolyi A., Pál G., Vincze C., Wappler E. A., Nagy Z., Lovas G. (2011) Transforming growth factor beta activation following focal ischemia in the rat brain. 8th IBRO World Congress of Neuroscience, Florence, Italy, D472.

16 Cservenák M., Bodnár I., Usdin T. B., Palkovits M., Nagy G. M., Dobolyi A. (2011) The TIP39-PTH2 receptor system: new components in the regulation of prolactin release in lactation. Magyar Farmakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok közös Tudományos Konferenciája, Pécs. Acta Physiologica 202:S684, P18-01.

17 Pál G., Vincze C., Wappler E.A., Nagy Z., Lovas G., Dobolyi A. (2011) Spatial and temporal patterns of induction of transforming growth factor betas following middle cerebral artery occlusion in rat. 15th Congress of the European Federation of Neurological Societies, Budapest.

18 Dobolyi A., Cservenák M., Usdin T. B., Palkovits M. (2011) Maternally activated posterior thalamic neurons possibly convey the suckling information towards hypothalamic centers. 93rd Annual Meeting of The Endocrine Society, Boston, USA. Endocr Rev 32: P3-235.

Conference attendance with full or partial support of OTKA NNF2 85612 (each participant had presentation as listed above):

XXI International Semmelweis Symposium, Budapest, 2012 - Árpád Dobolyi, Éva Rebeka Szabó, Melinda Cservenák, Gabriella Pál

8th Forum of European Neuroscience, Barcelona, Spain, 2012 – Melinda Cservenák

International IBRO Workshop, Szeged 2012 - Árpád Dobolyi, Éva Rebeka Szabó, Melinda Cservenák, Gabriella Pál

A Magyar Élettani Társaság, a Magyar Anatómusok Társasága, a Magyar Biofizikai Társaság és a Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság Kongresszusa, Debrecen, Hungary, 2012 - Éva Rebeka Szabó, Melinda Cservenák, Gabriella Pál

8th IBRO World Congress of Neuroscience, Florence, Italy, 2011 – Árpád Dobolyi, Éva Rebeka Szabó, Melinda Cservenák

Magyar Farmakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok közös Tudományos Konferenciája, Pécs, Hungary, 2011 – Melinda Cservenák

15th Congress of the European Federation of Neurological Societies, Budapest, Hungary, 2011 - Gabriella Pál, Gábor Lovas

93rd Annual Meeting of The Endocrine Society, Boston, USA, 2011 – Árpád Dobolyi

Scientific report

Transforming growth factor beta (TGF-β) proteins are multifunctional cytokines whose neural functions are increasingly recognized. Three separate genes encode TGF-β1, -β2, and -β3 (Lawrence, 1996; Roberts, 1998). TGF-βs affect cell proliferation, differentiation, and extracellular matrix formation in a variety of tissues (Burt and Law, 1994) by means of serine-threonine kinase domain-containing TGF-β receptors (Arighi et al., 2009; Attisano and Wrana, 2002). In the normal brain, TGF-β1 immunoreactivity is present in the epithelial and meningeal cells of the choroid plexus (Unsicker et al., 1991). We recently the expression of TGF-β1 in some restricted brain regions by *in situ* hybridization histochemistry (Vincze et al., 2010). TGF-β2 and -β3 were constitutively present in several brain regions as, confirmed at the mRNA and protein levels and their expression patterns greatly overlapped (Vincze et al., 2010). In the cerebral cortex, TGF-β2 expression was very intense in layer V Layers III and IV also contained TGF-β2 and –β3, respectively, while TGF-βs were absent in the caudate putamen (Vincze et al., 2010). Evidence of their involvement in the development and plasticity of the nervous system as well as their functions in peripheral organs suggested that they exhibit neuroprotective functions, too. Indeed, TGF-β expression is induced following a variety of types of brain tissue injury but the available knowledge was scarce as to the induction of specific subtypes and the mechanisms of induction. In the framework of the proposal, we presented evidence that endogenous TGF-β1, -β2 and -β3 are expressed in brain tissue following a ischemic lesion. However, significant differences exist between the spatial and temporal patterns of expression of TGF-β subtypes. The induction of TGF-β1-3 was examined in the rat after focal ischemia at 3h, 24h, 72h and 1 month after transient (1h) or permanent (24h) middle cerebral artery occlusion (MCAO) model of focal ischemia (Fig. 1) and in early postnatal mice following unilateral ligature of the carotid artery, an experimental

model of neonatal ischemia, using *in situ* hybridization histochemistry and quantitative analysis.

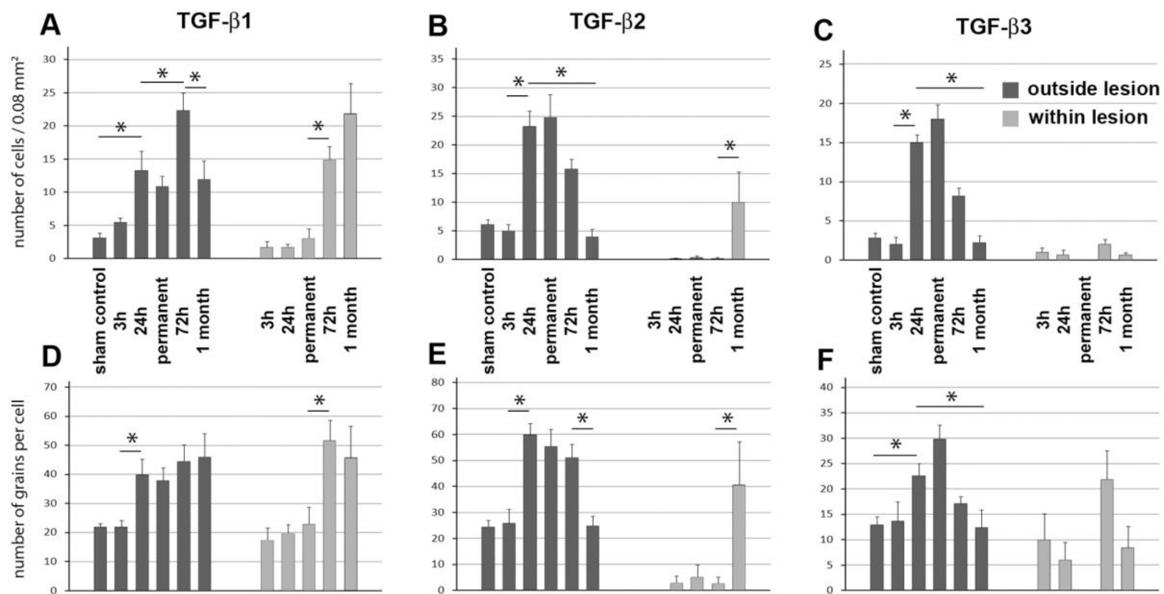

Fig. 1. The time course of TGF-β protein expression following MCAO. The numbers of cells within 200 x 400 μm areas (0.08 mm^2) of coronal brain sections in cortical layer II were counted immediately outside the lesion (dark gray) and 1 mm medial to the border of the lesion within the infarct area (light gray). The number of autoradiography grains was counted above the TGF-β-positive cell nuclei, indicated by autoradiography grain accumulation. Sections from at least 3 brains of the following groups of animals were included in the analysis: sham-operated rats 24 h following MCAO, rats at 3 h and 24 h following 1 h MCAO, rats with a permanently occluded middle cerebral artery 24 h following MCAO, and rats at 72 h and 1 mo following 1 h MCAO. A-C: The number of cells expressing TGF-β1, -β2, and -β3 in 0.08 mm^2. D-F: The number of autoradiography grains proportional to the mRNA levels of TGF-β1, -β2, and -β3 in single cells. Values at different time points were compared using one-way ANOVA followed by Bonferroni´s multiple comparisons tests for consecutive time points. The star symbol (*) indicates time points between which the number

of TGF-β-expressing cells or the mRNA level of the particular subtype of TGF-β in single cells significantly ($p < 0.05$) changed.

Double labeling with different markers was used to identify the localization of TGF-β mRNA relative to the penumbra and glial scar, and the types of cells expressing TGF-βs following MCAO (Fig. 2 and Table 1).

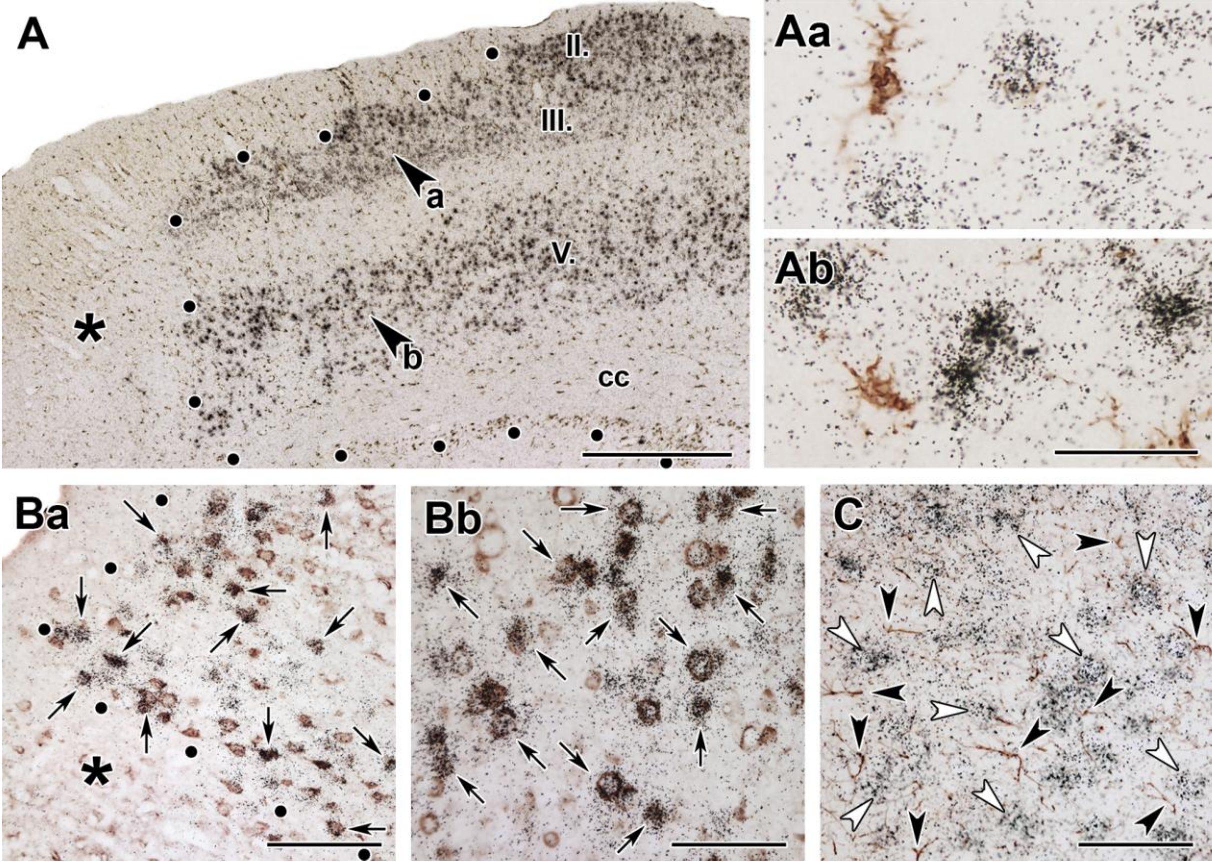

Fig. 2. TGF-β2 is induced in neurons but not in glial cells 24h after MCAO. **A**: Double labeling of TGF-β2 mRNA (black *in situ* hybridization signal) and immunoreactivity of the microglia marker Iba1 (brown precipitate). The lesion is indicated by star symbols (*) and the lesion border is demarcated by black dots. The positions of the high-magnification images in layer III and V of the cerebral cortex are indicated by black arrowheads as Aa, and Ab,

respectively. Iba1-immunoreactive microglia do not contain TGF-β2 mRNA in either layer of the cerebral cortex. **B**: Double labeling of TGF-β2 mRNA (black *in situ* hybridization signal) and NeuN-immunoreactive neurons (brown precipitate). Almost all TGF-β2 mRNA-expressing cells contained NeuN immunoreactivity in layer III (Ba), and in layer V (Bb). Examples of double-labeled cells are indicated by black arrows. **C**: TGF-β2 is not expressed in astrocytes. TGF-β2 mRNA expressing neurons are indicated by white arrowheads and GFAP-immunoreactive neurons are indicated by black arrowheads. There are no double-labeled cells present. Abbreviations: cc – corpus callosum. Scale bars = 1 mm for A, 50 µm for Ab, 200 µm for Ba, 100 µm for Bb, and 100 µm for C.

	TGF-β1				**TGF-β2**			**TGF-β3**
	layer II	**layer V**	**CP**		**layer II**	**layer V**		**layer II**
Number of TGF-β cells / 0.08 mm^2	18.3±4.9	16.7±3.5	20.5±4.5		18.3±4.2	17.0±3.9		14.0±4.5
Immunolabeled cells in the % of TGF-β cells								
NeuN	3.7±2.8	4.7±3.3	5.7±3.9		87,1±3,8	84,8±2,7		81,1±3.5
GFAP	26.3±2.8	21.6±6.2	22.2±4.4		4.2±2.3	4.0±2.1		9.3±5.8
Iba1	93.7±3.4	94.0±4.6	96.0±4.1		3.1±2.6	3.5±2.4		6.7±3.4
Hsp70	7.2±2.2	4.8±3.9	5.5±2.9		76.3±7.4	69.2±8.9		-
Fos	4.1±2.2	4.7±3.3	5.2±2.7		90.2±3.8	91.9±4.6		-
ATF-3	8.4±1.9	7.1±2.6	8.5±4.1		2.8±1.9	4.3±2.9		-

Table 1. Data on TGF- β1 is presented in 3 different locations, layer II and V of the cerebral cortex and the caudate putamen (CP). Because TGF-β2 is not expressed in the caudate

putamen, TGF-β2-expressing cells were only counted in the cerebral cortex. Similarly, data on TGF-β3 are provided only for layer II of the cerebral cortex. In the upper row, the total number of TGF-β -expressing cells counted in a 400 x 200 m rectangular-shaped area immediately outside the lesion is shown. The number of double-labeled cells was also counted in the same field, and the calculated ratios were averaged. One-way ANOVA did not indicate significant differences between brain regions for any of the markers.

TGF-β1 expression increased 3h after MCAO in the penumbra and was further elevated 24h after MCAO. TGF-β1 was present mostly in microglial cells but also in some astrocytes. By 72h and 1 month after the occlusion, TGF-β1 mRNA-expressing cells also appeared in microglia within the ischemic core and in the glial scar. In contrast, TGF-β2 mRNA level was increased in neurons but not in astrocytes or microglial cells in layers II, III, and V of the ipsilateral cerebral cortex 24h after MCAO. TGF-β3 was not induced in cells around the penumbra. Its expression increased in only a few cells in layer II of the cerebral cortex 24h after MCAO. The levels of TGF-β2 and -β3 decreased at subsequent time points. Permanent MCAO further elevated the levels of all 3 subtypes of TGF-βs suggesting that reperfusion is not a major factor in their induction. TGF-β1 did not co-localize with either Fos or ATF-3, while the co-localization of TGF-β2 with Fos but not with ATF-3 suggests that cortical spreading depolarization, but not damage to neural processes, might be the mechanism of induction for TGF-β2. The finding that TGF-β subtypes are expressed in separate cell types, and co-localize with different immediate early genes suggest that endogenous TGF-βs are induced by different mechanisms following an ischemic attack in the brain suggesting that they are involved in distinct spatially and temporally regulated inflammatory and neuroprotective processes. Furthermore, these results imply that the different subtypes of TGF-βs participate in different aspects of neural tissue protection (Pal et

al., 2012). Apart from neuroprotection following ischemia, TGF-βs were suggested to have neuroprotective actions in a variety of insults to the nervous tissue including trauma, sclerosis multiplex, neurodegenerative diseases, infections, and brain tumors. Different mechanisms might play a role behind the neuroprotective actions of different TGF-βs for the different disorders (Dobolyi et al., 2012). The anti-inflammatory action of TGF-βs is fully proved. A substantial amount of evidence is available to support the role of TGF-βs in scar formation including astrogliosis. TGF-β is known to affect cell survival, and an anti-apoptotic effect on neurons is supported by a number of experiments. A role of TGF-βs in excitotoxicity has also been intensively investigated. Similarly, the available evidence is strong for a role of TGF-βs to angiogenesis and their contribution to the vascularization of tumors. Finally, neuronal regeneration might also involve TGF-βs (Dobolyi et al., 2012).

Apart from a role of TGF-βs in brain pathologies, they were also shown to be involved in the physiological functions of the nervous system including neuronal differentiation and survival, synaptic transmission and plasticity, and neuroendocrine functions (Dobolyi, 2012). Our results pointed to a possible role of TGF-βs in maternal alterations (Cservenak et al., 2011). The expression of TGF-β1 is very similar to that of amylin in the preoptic area (Dobolyi, 2011), a brain region whose lesion leads to the elimination of maternal care (Numan, 1986). Amylin expression was also induced in maternally behaving (sensitized) non-lactating but not in non-sensitized nulliparous control females and Fos activation was demonstrated in amylin neurons in response to pup exposure in mothers (Fig. 3) suggesting that preoptic expression is related to maternal behavioral changes (Szabo et al., 2012).

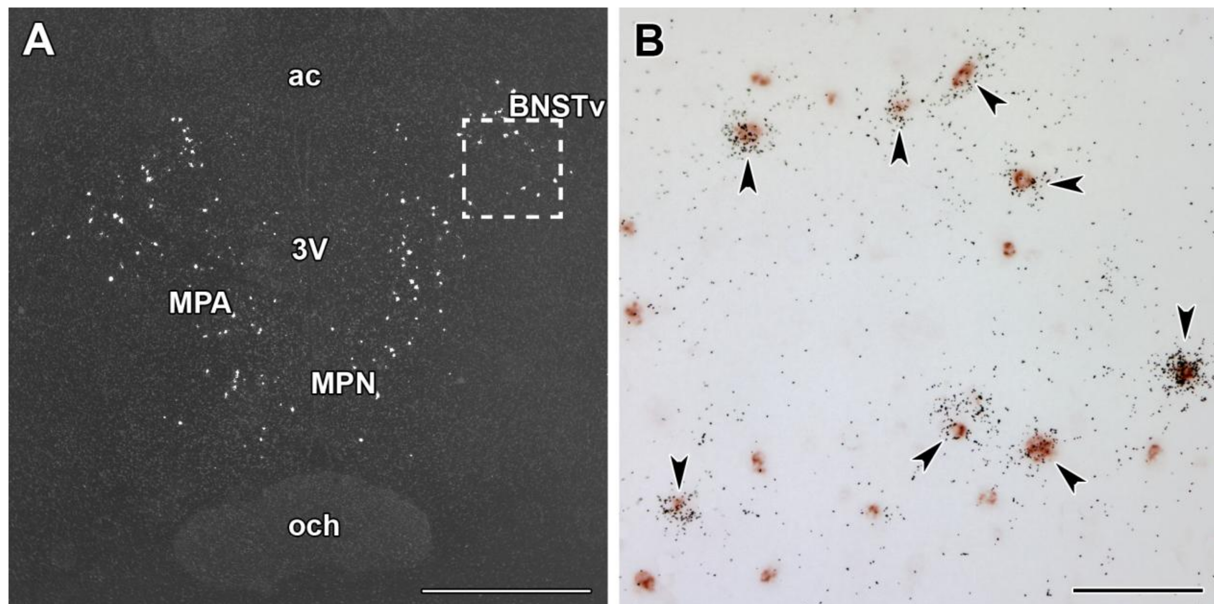

Fig. 3. Fos activation of amylin mRNA expressing neurons in response to pup exposure. **A**: A high density of amylin mRNA expressing neurons (white labeling) can be seen in a dark-field photomicrograph of the preoptic area in mother rat. **B**: The framed area in A is shown in bright-field picture at a high magnification. Most amylin mRNA-expressing neurons (situated below the black autoradiography dots) contained Fos-immunoreactivity (brown nuclei) in the preoptic area 2 h after pup exposure following 22 h separation. A considerable number of Fos-immunoreactive but amylin-negative neurons are also visible. Fos-immunoreactive neurons were present in the medial preoptic nucleus (MPN), the medial preoptic area (MPA) and the ventral part of the bed nucleus of the stria terminalis (BNSTv) with a distribution similar to that of amylin-expressing neurons. Additional abbreviations: ac – anterior commissure, och – optic chiasm, 3V – third ventricle. Scale bar = 1 mm for A and 100 µm for B.

However, we showed that TGF-β1 and amylin are not co-expressed by the same neurons. Furthermore, while amylin levels were decreased in mice lacking tuberoinfundibular peptide of 39 residues, a marker of neurons with ascending maternal input (Varga et al., 2012), TGF-β1 expression was not affected. Thus, although TGF-β1 is expected to play a role in central

reproductive regulation, it may not be involved in maternal regulations (Dobolyi, 2012). Instead, gonadotropin-releasing hormone neurons in the preoptic area known to contain TGF-β receptors respond directly to TGF-β1 stimulation (Prevot et al., 2000). Indeed, incubation of preoptic explants with TGF-β1 caused a significant, dose-dependent decrease in GnRH mRNA expression in individual neurons, which was inhibited by addition of the soluble form of TGFbeta-RII to the incubation medium (Bouret et al., 2004). These results support that TGFbeta1 may directly influence GnRH expression and/or secretion in vivo by acting on the perikarya of GnRH neurons (Dobolyi, 2012).

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Literature cited

Arighi CN, Liu H, Natale DA, Barker WC, Drabkin H, Blake JA, Smith B, Wu CH. 2009. TGF-beta signaling proteins and the Protein Ontology. BMC Bioinformatics 10 Suppl 5:S3.

Attisano L, Wrana JL. 2002. Signal transduction by the TGF-beta superfamily. Science 296:1646-1647.

Bouret S, De Seranno S, Beauvillain JC, Prevot V. 2004. Transforming growth factor beta1 may directly influence gonadotropin-releasing hormone gene expression in the rat hypothalamus. Endocrinology 145:1794-1801.

Burt DW, Law AS. 1994. Evolution of the transforming growth factor-beta superfamily. Prog Growth Factor Res 5:99-118.

Cservenak M, Bodnár I, Usdin TB, Palkovits M, Nagy GM, Dobolyi A. 2011. A new player in the regulation of suckling-induced prolactin release in rat. 8th IBRO World Congress of Neuroscience, Florence, Italy, C151.

Dobolyi A. 2011. Novel potential regulators of maternal adaptations during lactation: tuberoinfundibular peptide 39 and amylin. J Neuroendocrinol 23:1002-1008.

Dobolyi A. 2012. Transforming Growth Factor Beta in the Central Nervous System. In: Neuroscience – Dealing With Frontiers, Contreras CM, Ed, In Tech, Rijeka, Croatia, pp. 129-148.

Dobolyi A, Vincze C, Pal G, Lovas G. 2012. The neuroprotective functions of transforming growth factor Beta proteins. Int J Mol Sci 13:8219-8258.

Lawrence DA. 1996. Transforming growth factor-beta: a general review. Eur Cytokine Netw 7:363-374.

Numan M. 1986. The role of the medial preoptic area in the regulation of maternal behavior in the rat. Ann N Y Acad Sci 474:226-233.

Pal G, Vincze C, Renner E, Wappler EA, Nagy Z, Lovas G, 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.

Prevot V, Bouret S, Croix D, Takumi T, Jennes L, Mitchell V, Beauvillain JC. 2000. Evidence that members of the TGFbeta superfamily play a role in regulation of the GnRH neuroendocrine axis: expression of a type I serine-threonine kinase receptor for TGRbeta and activin in GnRH neurones and hypothalamic areas of the female rat. J Neuroendocrinol 12:665-670.

Roberts AB. 1998. Molecular and cell biology of TGF-beta. Miner Electrolyte Metab 24:111-119.

Szabo ER, Cservenak M, Dobolyi A. 2012. Amylin is a novel neuropeptide with potential maternal functions in the rat. FASEB J 26:272-281.

Unsicker K, Flanders KC, Cissel DS, Lafyatis R, Sporn MB. 1991. Transforming growth factor beta isoforms in the adult rat central and peripheral nervous system. Neuroscience 44:613-625.

Varga T, Mogyorodi B, Bago AG, Cservenak 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.

Vincze C, Pal G, Wappler EA, Szabo ER, Nagy ZG, Lovas G, Dobolyi A. 2010. Distribution of mRNAs encoding transforming growth factors-beta1, -2, and -3 in the intact rat brain and after experimentally induced focal ischemia. J Comp Neurol 518:3752-3770.