DOI:10.14753/SE.2012.1755

Neuroanatomy and function of brain structures involved in the regulation of prolactin secretion and milk yield

Ph.D. Theses

FLÓRA SZABÓ M.D.

Department of Human Morphology and Developmental Biology
Faculty of Medicine, Semmelweis University

János Szentágothai Doctoral School of Neuroscience
Program of Neuroendocrinology (6)

Tutor:	Prof. Katalin Köves M.D., D.Sc.
Referees	Associate Prof. Szabolcs Várbíró M.D., Ph.D. Prof. Klára Matesz, M.D., D. Sc.
Chairman of examining committee	Prof. János Sólyom, M.D., D.Sc.
Members of examining committee	Prof. Jenő Egyed, M.D., D.Sc. Associate Prof. Andrea Székely, M.D., Ph.D.

BUDAPEST, 2012

DOI:10.14753/SE.2012.1755

List of content

DOI:10.14753/SE.2012.1755

DOI:10.14753/SE.2012.1755

DOI:10.14753/SE.2012.1755

ABBREVIATIONS

ACTH	adrenocorticotrop hormone
ARC	arcuate nucleus
ATP	adenosine triphosphate
BDA	biotinylated dextran amine
Cas	catecholamines
CART	cocaine and amphetamine regulated transcript
CGRP	calcitotin gene related peptide
CNS	central nervous system
CTP	cytosine triphosphate
Cy2	cyanine dye excited at 492 nm, more photostable than FITC
Cy5	indodicarbocyanine dye excited at 650 nm, more photostable than rhodamine
D2	dopamine receptor 2
DA	dopamine
DOPA	dihydroxy phenilalanine
DAB	diamino benzidine tetrahydrochloride
DBH	dopamine-β-hydroxylase
DEPC	diethylpyrocarbonate
DYN	dynorphine
EDTA	ethylenediaminetetraacetic acid
ENK	enkephaline
FG	fluorogold
GAL	galanine
GH	growth hormone
GH_3	lactomammotrope cell line
GTP	guanine triphosphate
KPBS	potassium phosphate buffer-saline
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
ME	median eminence
NEDA	neuroendocrine dopaminergic
NMDA	N-metil-D-aspartate
OD	optical density
OXY	oxytocin
PCR	polymerase chain reaction
PFA	paraformaldehyde
PHDA	periventriculo-hypophysial dopaminergic
PPN	peripeduncular nucleus
PRL	prolactin
PRL-R	prolactin receptor
PV	paraventricular nucleus
PvG	paravertebral ganglion
RIA	radioimmunoassay
RT-PCR	reverse transcript-polymerase chain reaction

DOI:10.14753/SE.2012.1755

SIPR	suckling induced prolactin release
SPFpc	subparafascicular parvocellular nucleus
TH	thyrosin hydroxylase
TIDA	tuberoinfundibular dopaminergic
Th	thoracic
THDA	tuberohypophyseal dopaminergic
TIP-39	tuberoinfundibular peptide-39
TRNA	torula RNA
UTP	uracil triphosphate
VAChT	vesicular acethylcholine transporter
VLM	ventrolateral medulla
VMN	ventromedial nucleus
VR	ventral rootlet

DOI:10.14753/SE.2012.1755

1. **INTRODUCTION**

The offsprings of mammalian species are fed by milk produced in the mammary gland of their mother. Secretion of milk is a process called lactation. It has two major phases: 1. initiation of the milk secretion is the lactogenesis; 2. maintaining of the secretion is the galactopoiesis, in rats it is also called midlactation (see Tucker 1981).

The lactogenesis is further devided into stages I and II. *Stage I.* The mammary alveolar cells differentiate histologically and enzymatically. During this stage colostrum is formed. It is rich in fat, proteins and immunoglobulins. It does not contain lactose. In rats Stage I. begins 30 hours before delivery. *Stage II*. Copious amount of milk is produced. Begining of lactogenesis coincides with decline in the level of progesterone and transient elevation of estradiol, prolactin (PRL), growth hormone (GH), and glucocorticoids. In rats this stage occurs immediately prior to and at the time of parturition. Fig. 1. shows changes in the concentration of hormones in blood during periparturient period. In the Stage II. the milk contains proteins, fat, carbohydrates (lactose), minerals, and vitamins.

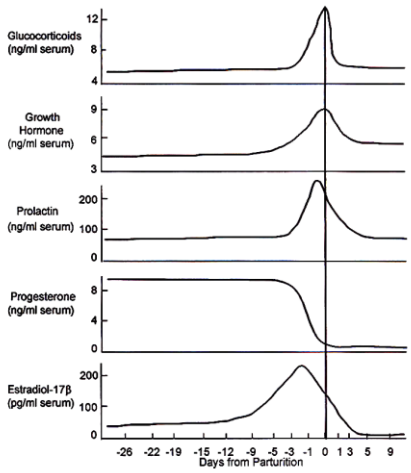

Fig. 1. Hormonal changes in the serum around parturition (Tucker HA. Neuroendocrinology in Physiology and Medicine, 2000, p. 169).

The production of milk is regulated by the above-mentioned hormones. Removal of milk happens in response to suckling (Hanwell and Linzell 1973). This neuroendocrine reflex mechanism is called milk ejection.

DOI:10.14753/SE.2012.1755

2. BACKGROUND

Because my theses include studies of brain structures regulating PRL secretion I will describe the biochemistry and receptors of PRL, distribution of PRL and its receptors and the function and regulation of this hormone.

2.1.Biochemistry of PRL

Rat PRL is a protein hormone composed of a polypeptide chain of 197 amino acids (Nicoll et al 1986) (Fig. 2). Its molecular weight is 23 kD. A 22 kD variants is also described (Antony et al 1993). PRL was first discovered in the pituitary gland (Stricker and Greuter 1928; Riddle et al 1932). The rat PRL was characterized by Parlow and Shome in 1976. It is encoded by chromosoma 17 (Alam et al 2006).

Fig. 2. Amino acid sequence of rat anterior pituitary PRL. A: alanin; C: cystein; D: aspartic acid; E: glatamic acid, F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophan; Y: tyrosine. Three disulfide bridges are found between cysteines. From: Neill, J.D. and Nagy, Gy.M. Prolactin secretion and its control (In The physiology of Reproduction, Eds: Knobil, E.K. and Neill, J.D., 1994).

DOI:10.14753/SE.2012.1755

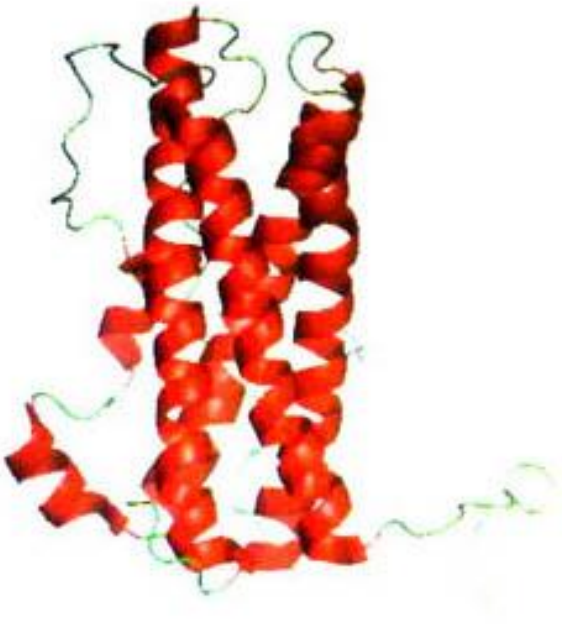

Fig. 3. Three dimensional image of PRL.

The three dimensional image of PRL molecule was constructed by Goffin and his coworkers (1995). The model contains the four-helix bundle and binding loops joining the helices.

Extensive posttranslational modifications of PRL have been reported. PRL may be cleaved, deamidated, phosphorylated, glycosylated, sulfated and disulfide dimerized (Sinha 1995).

In human serum two high molecular mass forms of circulating PRL were identified, macroprolactin (big-big prolactin) with 100kD MW or over and big prolactin with 40-60 kD MW (Fahie-Wilson et al 2005). Their clinical significance is not clarified but they may be responsible for hyperprolactinemia.

2.2. Structures producing PRL

PRL is secreted in various tissues. In the anterior pituitary acidophilic cells, known as mammotropes or mammosomatotropes, secrete PRL (reviewed by Frawley and Boockfor 1991). Fig. 4 shows the characteristic appearance of PRL immunoreactive cells in the anterior pituitary of a female rat.

DOI:10.14753/SE.2012.1755

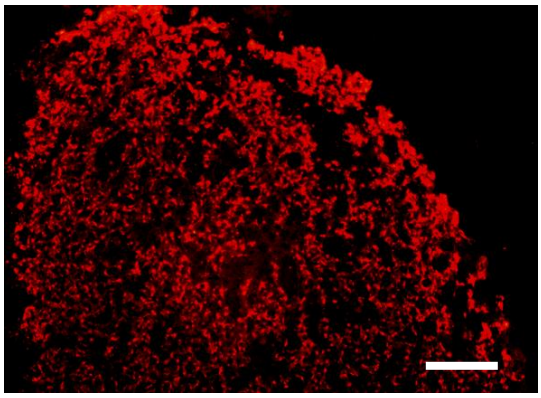

Fig. 4. Immunofluorescence staining shows PRL immunoreactive cells in the anterior pituitary of a diestrous female rat. The cells are evenly distributed in the anterior lobe and they show a cup-shaped appearance. Scale: 200 μm. (Courtesy of Andrea Heinzlmann, Assistant Professor, Department of Human Morphology and Developmental Biology.)

During lactation the mammotropic cells proliferate. They loose their cup-shaped appearance. Fig. 5 shows PRL staining of an anterior pituitary deriving from a midlactating rat (sacrificed 10 days after parturition).

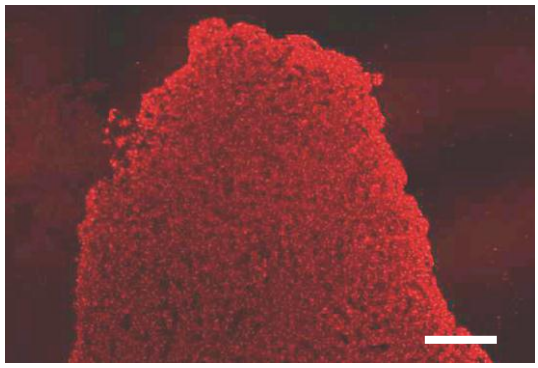

Fig. 5. Immunofluorescene staining showing PRL immunoreactivity in the anterior pituitary of a midlactating rat. PRL cells are extremly proliferated. Scale: 200μm.

In the last thirty years PRL and PRL-like immunoreactivities or PRL mRNA was demonstrated over ten tissues other than the pituitary gland (Sinha 1995). Table 1 shows the tissues where PRL or PRL-like molecules were demonstrated and the related references.

The richest source of peripheral PRL is the placenta. This molecule is called placental-lactogene produced by decidual cells (Golander et al 1978; Rosenberg et al 1980; Handwerger et al 1990; Alam et al 2008). PRL-like molecules were also demonstrated in

DOI:10.14753/SE.2012.1755

the endometrium, myometrium and smooth muscle fibroid of uterus (Masler and Riddick 1979; Walters et al 1983; Nowak et al 1993).

PRL mRNA was shown in immunocells including tymocytes, T-lymphocytes and T-lymphoblasts (DiMattia et al 1988; Montgomery et al 1992; Pellegrini et al 1992; De Bellis et al 2005).

Table 1. Tissues other than pituitary in which PRL-like molecules have been reported.

Occurrence of PRL	References
Placenta	Golander et al, 1978; Rosenberg et al, 1980 Handwerger et al, 1990; Alam et al, 2008
Uterus	Masler and Riddick, 1979; Walters et al, 1983; Eyal et al, 2007
Immune system	Di Mattia et al, 1988; Montgomery et al, 1992; Pellegrini et al, 1992; De Bellis et al, 2005
Mammary gland	Kurtz et al, 1993; Koizumi et al, 2003
*Adrenal gland**	Nolin, 1978
Corpus luteum	Nolin, 1978; Erdmann et al, 2007
Prostate	Harper et al, 1981; Nevalainen et al, 1997 Untergasser et al, 2001
Testes	Roux et al, 1985; Untergasser et al, 1996 Imaoka et al, 1998
*Urethral gland**	Tsubura, 1986
Lacrimal gland	Wood et al, 1999
*Sweat gland**	Roberstson et al, 1989
*Pancreatic islets**	Meuris et al, 1983
Brain	Fuxe et al, 1977; Harlan et al, 1989; Paut-Pagano et al, 1993; Wilson et al, 1992

- Biochemical or molecular biological evidence in tissues written by italic letters is not available.

DOI:10.14753/SE.2012.1755

The synthesis of PRL in mammary gland was first demonstrated by Kurtz and coworkers in 1993. PRL mRNA was localized in the epithelium of alveoli and ducts of lactating mammary gland by *in situ* hybridization. It suggests that the mammary gland might contribute to PRL in milk by de novo synthesis. Another research group was able to show PRL mRNA in the sebaceous glands of nipple (Koizumi et al 2003).

The presence of PRL in other peripheral organs including adrenal gland and the corpus luteum, uterus (Nolin et al 1978; Erdmann et al 2007; Eyal et al 2007), the prostate gland (Harper et al 1981; Nevalainen et al, 1997; Untergasser et al, 2001), testes (Roux et al, 1985; Untergasser et al, 1996; Imaoka et al 1998) and urethral gland (Tsubura et al 1986) lacrimal gland (Wood et al 1999), sweat gland (Robertson et al 1989), pancreatic islets (Meuris et al 1983) was also demonstrated but the presence of it in adrenal gland, urethral gland, sweat gland, pancreatic islets, and gut was not confirmed by the mRNA study.

PRL was discovered in the brain as well. The concentration of PRL in brain tissues much lower than in the anterior pituitary. Fuxe and his coworkers (1977) demonstrated at the first time that PRL-like immunoreactivity is present in some hypothalamic nerve terminals. The origin of these fibers were not explored by this research group. Twelve years later Harlan and his coworkers (1989) demonstrated PRL immunoreactive cell bodies in the hypothalamic arcuate nucleus (ARC) and in adjacent areas ventral to the ventromedial nucleus (VMN). Fiber projections extended rostrally to the anterior hypothalamus, preoptic area, nucleus accumbens, septum, diagonal band of Broca, caudate putamen, frontal cortex, and accessory olfactory bulb and to the central amygdala. Another team (Paut-Pagano et al 1993) published contraversary results. They have only found PRL immunoreactive cell bodies in the lateral hypothalamic area surrounding the fornix, not in the ARC; however, fibers dispersed all over the brain. After hypophysectomy amount of PRL was not decreased indicating that PRL demonstrated in the brain areas is produced locally. With the use of reverse transcript-polymerase chain reaction (RT-PCR) it was shown that PRL mRNA in the brain is identical to anterior pituitary PRL mRNA (Wilson et al 1992).

DOI:10.14753/SE.2012.1755

2.3. PRL receptor (PRL-R)

PRL-Rs belong to cytokine receptor family (Cosman et al1990). They have three domains: a ligand-binding extracellular, a single hydrophobic transmembrane, and a cytoplasmic domain. In rats two isoforms are described, short and long forms with 291 and 592 amino acids, respectively (Kelly et al 1993). The two isoforms differ in the length of intracellular domain. Intracellular domain is necessary for signal transduction. The two isoforms are the products of a single gene and they are generated by alternative splicing. PRL-R is encoded by chromosome 2q16. Signal tranduction pathway is the tyrosine kinase Jak-2 system.

PRL-Rs are widely distributed in rat tissues. With the use of quantitative PCR technique Nagano and Kelly (1994) mapped the distribution of PRL-R isoforms in 17 tissues (cerebral cortex, choroid plexus, hypothalamus, pituitary, heart, lung, thymus, spleen, liver, pancreas, kidney, adrenal, ovary, uterus, skeletal muscle, skin and mammary gland) of adult female rats during estrous cycle, pregnancy and lactation. In the hypothalamus and pituitary both receptors are expressed but the predominant form is the long isoform. It was also seen that the amount of receptors is higher in diestrous than in proestrous stage of the cycle. On the contrary, in the cerebral cortex and the peripheral reproductive organs the amount of the long form was higher in proestrus than in diestrus. The liver is the only organ where the short form is clearly dominant. In the pancreas a high level of PRL-R was found in the islets. The first immunohistochemical detection of the PRL-R immunoreactivity (Roky et al 1996) revealed that the receptor is associated with nerve cell bodies where a dense PRL immunoreactive fiber staining was found earlier (Fuxe et al 1977). PRL-R-like immunoreactive neurons were found in pyramidal cell layer of the cerebral cortex, septal nuclei, amygdaloid complex, hypothalamic nuclei (suprachiasmatic, supraoptic, paraventricular and dorsomedial), substantia nigra, habenula and in the subcommissural organ.

Mammary gland is the primary target tissue for PRL action. Both at the end of pregnancy and on day 7 of lactation the long form was expressed 6-10 fold more than the short form. During pregnancy and lactation the level of the long form was maintained at a relatively high level.

Immunohistochemistry revealed that the PRL-Rs are associated with all endocrine cell populations in the anterior pituitary (Morel et al 1994). The highest level of receptors was found on somatotropes, and decreasing number on lactotropes, then thyrotropes, corticotropes and gonadotropes. These findings suggest that PRL influences the pituitary hormone secretion via auto- and paracrine manners.

With RT-PCR, Northen and Western blot analysis the presence of PRL-Rs was revealed in gonads of both sexes (Zhang et al 1995; Guillaumot and Benahmed 1999), and *in situ* hybridization identified receptors on the interstitial Leydig cells, Sertoli cells and on the spermatogenetic cell line as well (Zhang et al 1995; Hondo et al 1995). *In situ* hybridization also revealed PRL-Rs in the acinar epithelium and in the interstitium of the lacrimal gland (Wood et al 1999). In the liver PRL-Rs and their regulation by sexual steroids were also demonstrated (Tanaka et al 2005).

2.4. Functions of PRL

Posttranslational modification gives rise to different variants of PRL molecules (Fig. 6) which have different functions when bind to the receptors.

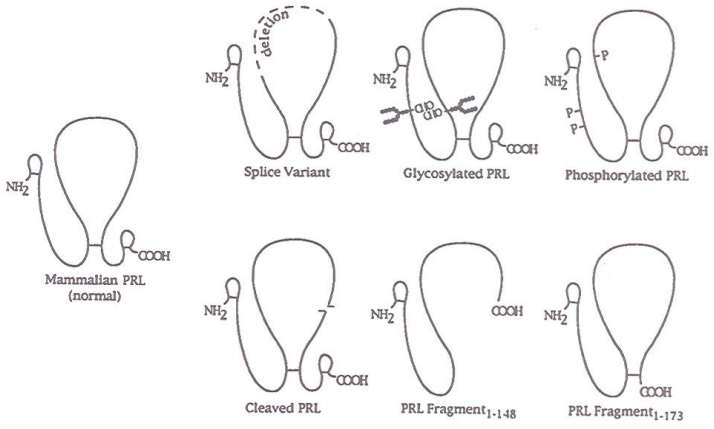

Fig. 6. Schematic diagram of PRL molecule and some structural variants. CHO refers to the N-linked carbohydrate moieties. P represents the site of phosphorilation. Broken line indicates the delition of amino acid residues. The nick in the large disulfide loop shows a proteolytic cleavage site. From Sinha, Y.N. Structural variants of prolactin: Occurrence and physiological significance. Endocrine Reviews 16: 358, 1995.

Most throughly investigated variant is the short PRL fragment (1-148). It lacks the fourth helix. This is a potent inhibitor of capillary endothelial cell proliferation (Ferrara et

al 1991). Cleaved PRL increased thymidine incorporation into DNA in gonadotropes and thyrotropes, but not in other pituitary cell types. This results indicate that cleaved PRL is a potent paracrine growth regulator in the pituitary tissue (Andries et al 1992). Phosphorylated PRL inhibited the secretion of nonphosphorylated PRL from a lactomammotrope cell line (GH_3). In this way it serves as autocrine regulator of PRL secretion (Ho et al 1989). PRL complexed with immunoglobulins produces growth response in peripheral blood lymphocytes. Similar growth promoting effect of PRL-immunoglobulin complex was demonstrated in malignant B-lymphocytes (Walker et al 1995). It means that the modified PRL may play a role as growth factor for these cells (Walker et al 1993).

PRL knock-out in mice induces infertility, but does not prevent the maternal behavior. In the mammary gland a normal ductal tree develops, but the ducts fail to develop lobular decorations, which is characteristic of the normal virgin adult mammary gland (Horseman et al 1997).

2.5. Regulation of PRL secretion

It was demonstrated by Everett (1954, 1956) more than fifty years ago that a pituitary autograft without hypothalamic connections can maintain the pseudopregnancy and corpora lutea. He postulated the existence of a hypothalamic factor which was released into the portal blood and inhibited the PRL secretion. A few years later Talwaker in Meites' laboratory (Talwalker at al 1963) confirmed the existence of a PRL inhibiting factor in hypothalamic extracts. Soon it was realized that this inhibiting factor was dopamine (DA) (Macleod 1974).

DA is one of catecholamine neurotransmitters. There are neurons which use it as neurohormone. These neurons take up tyrosine and tyrosine hydroxylase (TH) converts it into dihydroxy-phenilalanine (DOPA). This is the immediate precursor of DA. This is further converted into DA by aromatic L-amino acid decarboxylase. In other neurons DA serves as precursor for synthesis of norepinephrine.

DOI:10.14753/SE.2012.1755

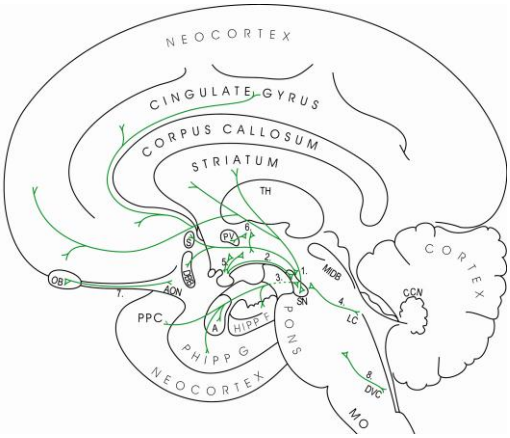

Figure 7. Schematic illustration of the dopaminergic neurons and their projections to various parts of the central nervous system. 1. Meso- (nigro-) striatal pathway. 2. Meso- (nigro-) hypothalamic-median eminence pathway. 3. Ventral mesolimbic pathway. 4. Mesopontine pathway. 5. Tubero-infundibular (TIDA) and tubero-hypophyseal pathway (THDA). 6. Incertohypothalamic pathway. 7. Olfactory bulb (probable projection to AON). 8. Dopaminergic fibers innervating the dorsal vagal complex. Abbreviations: A = amygdala; AON = anterior olfactory nucleus; CCN = central cerebellar nuclei; DBB = diagonal band of Broca; DVC = dorsal vagal complex; HIPP F = hippocampal formation; LC = locus coeruleus; MIDB = midbrain; MO = medulla oblongata; OB = olfactory bulb; PHIPP G = parahippocampal gyrus; PPC = prepiriform cortex; PV = hypothalamic paraventricular nucleus; S = septum; SN = substantia nigra; TH = thalamus. From: Köves and Heinzlmann, Neurotransmitters and Neuropeptides in Autism (in: New Autism Research Developments, Ed.: B. S. Mesmere, 2008, p. 35)

DA is produced in several brain regions such as substantia nigra, zona incerta, olfactory bulb and the hypothalamic ARC. Fig. 7 schematically illustrates the most important dopaminergic pathways in the rat central nervous system.

The ARC begins just rostral to the hypothalamic infundibular recess and lies along the walls of this recess troughout its length. Two distinct subdivisions are generally recognized: dorsomedial part which has a small cell population and the ventrolateral part which has medium-sized neurons (Meister and Hökfelt 1988; Simerly and Young 1991). Fig. 8 shows the ARC at rostral (A) and caudal (B) levels by cresyviolette staining.

DOI:10.14753/SE.2012.1755

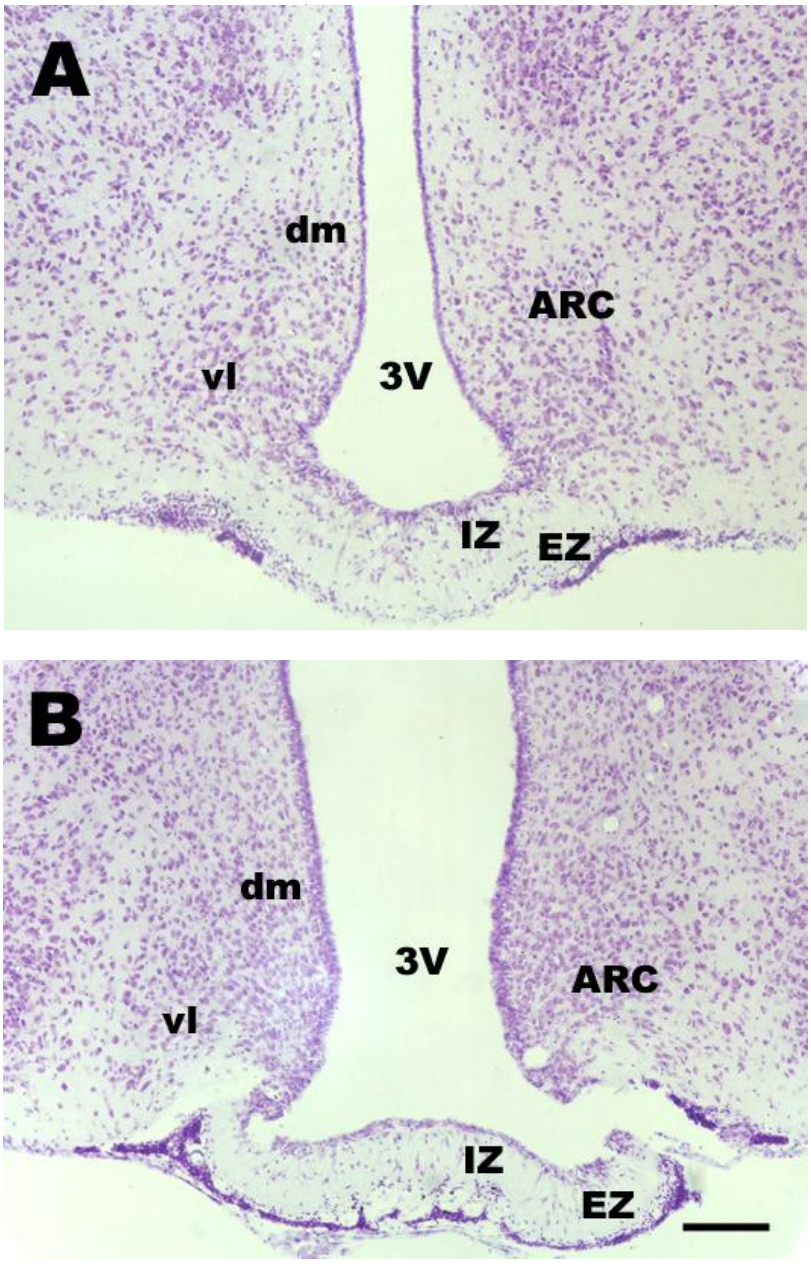

Fig. 8. Microphotographs demonstrating ARC in the frontal sections of a midlactating rat hypothalamus at two rostrocaudal levels. **A** is at A6,8 and **B** is at A5,8 to interaural line. Cresyviolette staining. Abbreviations: 3V = third ventricle, ARC = arcuate nucleus; dm = dorsomedial part of ARC; EZ = external zone of the median eminence; IZ = internal zone of the median eminence; vl = ventrolateral part of ARC. Scale: 200 μm.

DOI:10.14753/SE.2012.1755

The hypothalamic neuroendocrine dopaminergic (NEDA) neurons are involved in the regulation of PRL secretion. Three populations are identified to the rostro-caudal direction: 1. the periventriculo-hypophysial dopaminergic (PHDA), 2. the tubero-hypophyseal dopaminergic (THDA) and 3. the tubero-infundibular dopaminergic (TIDA) systems. The cells of origin of these pathways are located in the periventricular-ARC region. PHDA neurons are located in the most rostral subdivision and terminate in the intermediate lobe. THDA neurons occupy the middle region and terminate in both intermediate and neural lobes. TIDA neurons are located in the middle and posterior subdivisions and terminate around the capillaries in the external zone of the median eminence (ME) (see Freeman et al 2000; Tóth et al 2002).

Fig. 9 schematically illustrates PHDA, THDA and TIDA neurons and their termination in the pituitary gland and in external zone (EZ) of ME. PHDA neurons terminate in the intermediate lobe, THDA neurons in both neural and intermediate lobes, and TIDA neurons in EZ of ME.

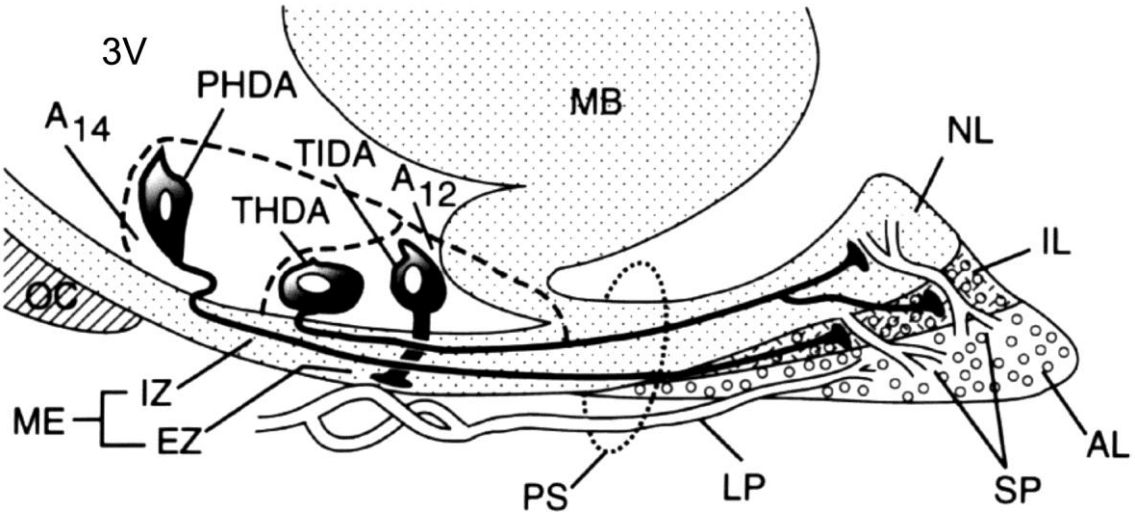

Fig. 9. Schematic illustration of PHDA, THDA and TIDA pathways in the sagittal section of the hypothalamus and the pituitary gland (From Freeman, Kanyicska, Léránth and Nagy: Physiological Review 80, 1523-1631, 2000). Abbreviations: 3V = third ventricle; A12 and A14 = dopaminergic cell groups; AL = anterior lobe; EZ = external zone; IL = intermediate lobe; IZ = internal zone; LP = long portal vessels; MB = medial basal hypothalamus; ME = median eminence; NL = neural lobe; OC = optic chiasm; PS = pituitary stalk; SP = short portal vessels.

DOI:10.14753/SE.2012.1755

Fig. 10 shows TH immunostaining in ARC at mid antero-posterior level (A6,4 to interaural line).

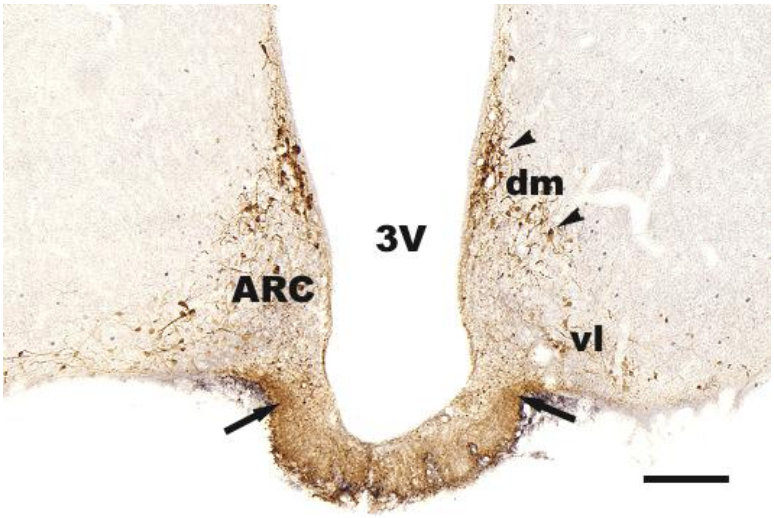

Fig. 10. TH immunostaining in ARC nucleus. TH immunopositive cells are mainly located in the dorsomedial part of ARC. In the ventrolateral part of this nucleus the TH cells are scattered. TH cells located in the dorsomedial part of ARC project to the ME forming the tubero-infundibular dopaminergic pathway. They are called TIDA neurons. In ME the dopaminergic fibers are denser in the lateral part of the infundibular recess then in the middle portion and located in the external zone of the ME. Arrowheads show TIDA neuronal cell bodies, arrows show TIDA fibers in the external zone. Abbreviations: 3V = third ventricle; ARC = arcuate nucleus; dm = dorsomedial part of ARC; vl = ventrolateral part of ARC. Scale: 250μm.

Under non-lactating conditions, these neurons produce DA and continuously and tonically release it into the hypophysial portal circulation (Ben-Johnatan 1977). DA acts on D2 receptors of lactotropes to inhibit PRL release. When DA release is inhibited, PRL is rapidly released into the general circulation (Ben-Jonathan and Hnasko 2001). PRL can be controlled in this manner by a host of stimuli such as stress, sexual activity and stimuli to the breast (Pena and Rosenfeld 2001).

There are ample evidence that suggest that afferent activity to the TIDA neurons is a powerful regulator of TIDA neuronal activity and thus, of PRL secretion. Mammary stimulation becomes especially critical during lactation for maintaining milk production via its ability to release PRL (Whitworth and Grosvenor 1984). Studies of electrical stimulation of the mammary nerve of lactating rats reveal that a 3 minute stimulation

DOI:10.14753/SE.2012.1755

produces a 63% decline in pituitary stalk and ME DA levels preceding the rise in plasma PRL (De Greef et al 1981; Plotsky and Neill 1982; Plotsky et al 1982). Further evidence to support the importance of afferent activity to the TIDA neurons is the observation that prevention of suckling on teats of only one side up-regulates TH expression in TIDA neurons on the contra-lateral side to blocked nipples (Berghorn et al 2001). This indicates that the sensory stimulus prompted by suckling is responsible for the TH suppression in TIDA neurons.

In the past years a lot of attention has been focused on the importance of suckling for successful lactation. There have been numerous debates about the ideal duration and frequency of breastfeeding episodes to ensure adequate milk supply. Over a million infant deaths have been attributed to the lack of breastfeeding in the world (McVea et al 2000), so in recent years there has been an increase in movements that advocate breastfeeding. Understanding the circuits that are responsible for this process is critical in understanding the physiological changes that take place in the body and their impact on maternal and infant health. This could also provide an explanation to how certain central nervous system (CNS) disorders such as tumors, head injury, infection (tuberculosis, histoplasmosis), or infiltrative diseases (sarcoidosis, hemochromatosis, lymphocytic hypophysitis) disrupt the process of lactation (Pena and Rosenfeld 2001).

The most widely studied neuroendocrine reflex responsible for milk production is the suckling induced PRL release (SIPR). It is clear that PRL secretion and release by mammotropes in lactating rats are mainly controlled by dopaminergic neurons of the medial basal hypothalamus (Leong et al 1983). DA acts as the main inhibitory transmitter, responsible for tonically inhibiting PRL production and release in non-lactating rats. At the beginning of lactation, suckling stimuli by the pups eventually reach the hypothalamus, inhibiting the activity of TIDA neurons which form one of catecholaminergic cell groups (Fuxe and Hökfelt 1972), thus allowing the release of PRL from the pituitary into the general circulation and in turn, PRL stimulates milk secretion.

The exact pathway from the nipples to the neurons of the medial basal hypothalamus that conveys the suckling stimulus to the TIDA neurons is not well characterized. Suckling also stimulates oxytocin (OXY) release from the magnocellular

supraoptico-paraventriculo-hypophyseal system. Previous reports have suggested that the release of PRL and OXY during suckling are coordinated (Samson et al 1986). By monitoring milk let-down reflexes due to OXY release or electrical activity of identified OXY neurons after brain stimulation or following suckling after lesions, a profile of brain sites involved in the suckling induced neuroendocrine axis has emerged. Studies indicate that the suckling stimulus from the mechanoreceptors of the nipples is delivered to the spinal cord with a relay in the cervical spinal nucleus (Dubois-Dauphin et al 1985). After ascending from this nucleus, a projection to the mesencephalic tegmentum (Dubois-Dauphin et al 1985; Hansen and Kohler 1984; Tindal and Knaggs 1971; Tindal and Knaggs 1969) rather than the more classical thalamic sites (Dubois-Dauphin et al 1985) conveys suckling signals to the hypothalamus for milk ejection control. There appears to be at least one additional relay before hypothalamic neuroendocrine neurons are reached.

The peripeduncular nucleus (PPN), nestled among the medial geniculate nucleus, the posterior intralaminar thalamic nucleus and the cerebral peduncle, has been suggested to be an important mediator of the suckling stimulus for successful lactation. Such observations were made based on studies in which these areas were lesioned (Factor et al 1993, Hansen and Kohler 1984) and lactation was impaired. Experiments using stimulation paradigms (Tindal and Knaggs 1969; 1975) noted that the lateral-most region of the midbrain tegmentum, likely within the PPN as defined by commonly used atlases (Paxinos and Watson 1986) were effective in releasing PRL. Previous studies (Tindal et al 1969) had determined that electrical stimulation of the more medial parts of the midbrain tegmentum also released PRL, but it is unclear whether that is due to stimulation of fibers of passage or of neurons. To resolve this, it is important to distinguish the PPN from the subparafascicular parvocellular nucleus (SPFpc) or more medial regions of the tegmentum.

An interneuronal relay from the mesencephalon to the hypothalamus is proposed, but has yet to be identified for either projection to OXY or TIDA system. Since the suckling stimulus excites OXY neurons, but inhibits TIDA neurons, the pathways must diverge somewhere and based on lesions, this divergence takes place upstream from the

midbrain site. It is likely that the signals travel together until they reach the brain stem where the neuronal pathways for milk ejection and PRL regulation diverge.

In a previous study suckling stimulus induced c-Fos expression in the ventrolateral medulla (VLM), locus ceruleus, lateral parabrachial nucleus, lateral and ventrolateral portions of the caudal part of the periaqueductal gray matter, and caudal portion of the paralemniscal nucleus (Li et al 1999b). This experiment also suggest the role of brain stem structures in relaying the suckling stimulus to the hypothalamus. In another study by the same research group it was found that fluorogold (FG) tracer injected in the ARC was retrogradely transported to the midbrain. The tracer appeared in some cell groups in which c-Fos was activated by suckling stimulus. These cell groups were mainly found in the PPN and VLM (Li et al 1999a). In this latter study the tracer spred over the border of the ARC. It is not sure that the neurons in the PPN and VLM project directly to the ARC.

It was observed that soon after the initiation of suckling, DA turnover and release are markedly reduced (Demarest et al 1983; Mena et al 1976; Merchenthaler 1993; Selmanoff and Wise 1981). Overall, inhibition of the TIDA system assumes the dominant feature during suckling via marked down-regulation of the rate limiting enzyme for DA synthesis, TH (Wang et al 1993). However, the expression of TH mRNA in TIDA neurons seems to be very dynamic, reflecting the changes in suckling activity. Previous studies determined that within 1.5 hrs of termination of suckling, the TIDA neurons showed early signs of up-regulation of TH mRNA reflected by the appearance of 1 or 2 sites of heteronuclear RNA in the nucleus of TIDA neurons (Berghorn et al 2001). An increase in cytoplasmic TH mRNA was seen about 6 hours after the termination of suckling (Berghorn et al 2001) and mRNA levels peaked by 12-24 hr. Evidence of increased protein synthesis was also noted in ME terminals at 6 hr (Berghorn et al 1995). From these data, it is uncertain if the early signs of up-regulation of TH represent a trigger for full up-regulation of TH mRNA or whether continuous stimulation of these neurons is necessary to achieve high TH levels.

Another peptide whose expression varies in TIDA neurons under non-lactating and lactating conditions is enkephaline (ENK). ENK is barely detectable in cycling rats,

while its levels dramatically increase in the ARC and ME of lactating animals (Ciofi et al 1993; Merchenthaler 1993). The data in the literature indicate that this up-regulation of ENK is due to the hyperprolactinemia of lactation (Merchenthaler 1994; 1995).

It is not clear what role ENK in TIDA neurons plays during lactation. Although existing data show that the TH producing activity of TIDA neurons is definitely suppressed, this does not mean that these neurons are not active in synthesizing other transmitters. Inactivity of the TIDA neurons during lactation would not allow existing DA to be released at all. However, although TH expression is low during suckling, some TH is still present in the cells and in the ME (Wang et al 1993) and thus some DA release is possible (Ben-Johnatan et al 1980). A study (Arbogast and Woogt 1998) has proposed that ENK could be co-released with DA and serve to attenuate the effect of DA on lactotropes, raising the possibility that ENK also contributes to PRL secretion. Other neuroendocrine systems could also be targets of ENK produced in the ARC. During suckling a number of neuroendocrine systems operates differently than in a cycling animal: among others, luteinizing hormone (LH) control changes dramatically and stress responses are greatly attenuated. ENK has been suggested to play a direct role in the regulation of luteinizing hormone-releasing hormone (LHRH) secretion by innervation of LHRH neurons. Recently Pimpinelli and his coworkers [2006] were able to demonstrate δ-opioid receptors in a subpopulation of LHRH nerve terminals of the ME. δ-opioid receptors can bind ENK suggesting that ENK fibers directly innervate LHRH nerve terminals. The data for the role of ENK in the regulation of LHRH and LH secretion and release are controversial. In *in vitro* studies, ENK inhibited the release of LHRH from mediobasal hypothalamus fragments (Drouva et al 1981; Higuchi and Kawakami 1981; Motta and Martini 1982; Moult et al 1981). Other studies show that opiate antagonists stimulate LH secretion in rats (Blank et al 1980; Cicero et al 1979; Higuchi and Kawakami 1981), sheep (Schillo et al 1985), primates (Gosselin et al 1983; Van Vugt et al 1983; Van Vugt et al 1984), and humans (Grossman et al 1981; Moult et al 1981). Controversial to this are the studies showing that the administration of ENK increases LH in male and female rats (May et al 1979; Motta and Martini 1982) and ENK stimulates

LH secretion in anterior pituitary cell cultures (Slama et al 1990). Therefore, the role of ENK in the regulation of LHRH or LH still needs to be further studied.

Several neuropeptides and neurotransmitters were identified in the cell groups which are the potential relay stations of the SIPR and not yet proved. In the ventrolateral part of ARC pro-opiomelanocortin (POMC), dynorphin (DYN), alpha-melanocyta stimulating hormone (α-MSH), cocaine and amphetamine regulated transcript (CART) and acetylcholin was also demonstrated in neuronal cell bodies. Recently it was shown that DYN is present in about 30% of POMC neurons (Maolood and Meister 2008) and 20% of POMC neurons also contain pituitary adenylate cyclase activating polypeptide (PACAP) mRNA (Dürr et al 2007). SPFpc was demostrated as the main source of the tuberoinfundibular peptide (TIP39) (Dobolyi et al 2003) which was isolated from the tuberoinfundibular region (Usdin et al 1999). In this region cell bodies were not identified, but the most dense TIP39 fiber network was shown here (Dobolyi et al 2003). Calcitonin gene related peptide (CGRP) cell bodies were also demonstrated in the SPFpc and PPN (Yasui et al 1989). In this region there is dense galanin (GAL) and ENK fiber networks which are termination of ascending pathways from the lumbar spinal cord (unpublished data).

The role of a serotoninergic mechanism located in the parvocellular portion of PV in the regulation of SIPR was also suggested by Bodnár and her coworkers (2002). They found that frontal cuts in front and behind the hypothalamus blocked SIPR. Administration of a serotonin blocker (5, 7-dihydroxitriptamin) into PV or the lesion of its parvocellular part prevented the SIPR. However, 6-hydroxidopamine (β-adrenerg blocker) administration into PV had no effect.

2.6. Autonomic innervation of the mammary gland

The nipple and the mammary gland receive not only sensory, but autonomic innervation as well. As it was discussed in details milk secretion is iniciated by PRL release and milk ejection is induced by OXY release (Bisset et al 1967; Mena et al 1979; Song et al 1988); however, the milk yield at the beginning of the suckling is basically influenced by noradrenergic input. It was demonstrated by Findlay and Grosvenor (1969)

that catecholamines depressed the milk yield at the beginning of suckling antagonizing the effect of OXY. It was also shown that β-adrenergic blocker propranolol given *icv* enhanced the milk yield (Morales et al 2001). It was supposed that the β-adrenergic blocker relieved the effect of OXY on the ductal constriction in the mammary gland. Trans-section of the spinal cord between thoracic (Th)3 and Th4 segments or pharmacological sympathectomy, but not adrenalectomy and hypophysectomy, results in a faster rate of milk flow (Mena et al 1979; 1995; Morales et al 2001). The above-mentioned results suggest that the inhibition of milk yield at the beginning of suckling is mediated by a reflex pathway closed in the central nervous system. The afferent limb may be the same as the SIPR pathway, the efferent limb may be provided by the sympathetic pre- and postganglionic neurons present in the intermedio-lateral cell-column in the spinal cord and in the sympathetic trunk, respectively. The central synaptic station or stations may be in the brain stem and in the hypothalamus, probably in PV.

It is well known that the postganglionic parasympathetic nervous system consists of cholinergic neurons. Cholinergic neurons also comprise a small population of sympathetic postganglionic neurons that innervate the sweat glands (Landis and Fredieu 1986; Schäfer et al 1997). It was demonstrated a few years ago that the vast majority of sympathetic postganglionic neurons innervating the porcine mammary gland were located in Th10 and Th11 sympathetic ganglia (Franke-Radowiecka 2007). Description of the autonomic innervation of the rat mammary gland was published by Gerendai at al (2001). It is not clarified at this moment what kind of neurotransmitters mediates the sympathetic stimulus to the alveoli and ductal wall of the mammary gland, which is cholinergic and which is adrenergic.

DOI:10.14753/SE.2012.1755

3. AIM OF EXPERIMENTS

3.1. Morphological studies on SIPR pathway

The aim of these morphological studies was to further clarify which of the mesencephalic nuclei provides a relay to the ARC or to a cell group in the vicinity of ARC. We conducted a series of tract tracing studies in non-lactating rats to determine if any direct PPN to ARC connections existed and if not, where the relay from the mesencephalon to the ARC neurons was located. The neurochemical nature of those arcuate neurons which receive ascending fibers from the mesencephalon and those which send fibers from the mesencephalon to ARC was also investigated.

3.2. Physiological studies

The aim of the physiological studies was to compare the dynamics of changes in the expression of TH and ENK mRNA in TIDA neurons following a brief interruption of suckling (3-4 hours). We wished to elucidate 1) whether such brief interruption triggers full TH up-regulation that continues for a time after pup return then it declines (Hypothesis I) or 2) whether reinitiation of suckling immediately stops this process of up-regulation as a switch (Hypothesis II) (Fig. 11).

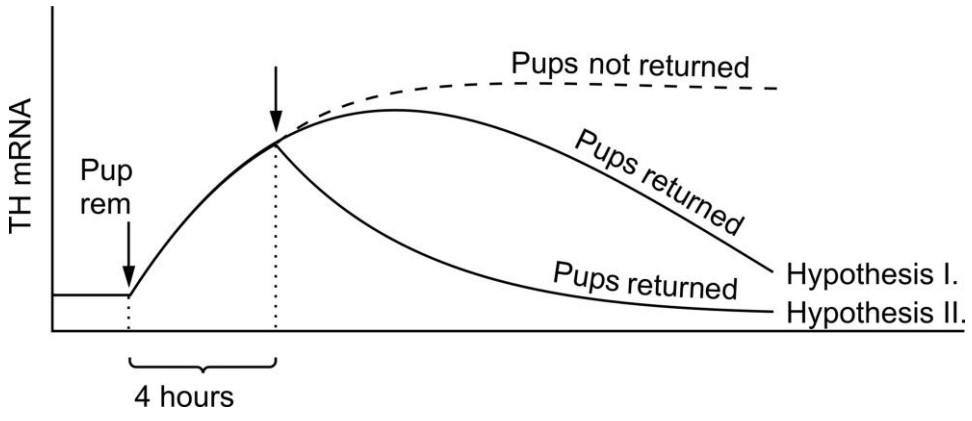

Fig. 11. Diagram showing our Hypothesis I and II. Pup rem = pup removal. Arrow indicates the time of the pup-return 4 hrs after pup removal.

DOI:10.14753/SE.2012.1755

We also planned to investigate 3) whether the time course of changes of ENK expression shows an opposite pattern as that of TH mRNA expression in the same animals and 4) whether the changes in the ENK expression well correlate with the ENK protein synthesis.

The progression of TH expression was followed up to 24 hrs after pups were returned to their dams. The dynamic changes in TH mRNA of TIDA neurons were compared with those of dams whose pups were permanently removed. In the same experimental animals we also investigated the ENK mRNA levels and followed the changes in the ENK peptide in the ME. Two more additional groups were included in this latter experiment. The animals of these groups were sacrificed 48 and 72 hrs after the removal of pups.

3.3. Studies on autonomic innervation of the mammary gland

The aim of this part of the work was to further explore the multisynaptic autonomic neuronal chain that innervates the nipples and the mammary glands of lactating rats using retrograde virus labeling and to chemically characterize the neurons of the neuronal chain which may participate in the regulation of the milk yield at the beginning of suckling.

DOI:10.14753/SE.2012.1755

4. MATERIALS AND METHODS

4.1. Animals

Sprague-Dawley female rats purchased from Zivic-Miller Laboratories, Inc. (Zelienople, PA) or Sprague-Dawley and Wistar female rats purchased from Gödöllő were used for the experiments. The University of Maryland's committee on Animal Care and Use approved the experimental paradigms according to NIH guidelines for the non-transsynaptic tracing and physiological experiments. The treatment of the animals using for virus labeling was in accordance with the rules of the „European convention for the protection of vertebrate animals used for experimental and other scientific purposes", Strasbourg, 1986. Our protocol was approved by the Department of Animal Health Care, permission number: 22.1/1158/3/2010. The rats (3-4 months old) were housed on a 12 hour light/12 hour dark schedule and given free access to food and water. Temperature was maintained at 22±2°C.

4.2. Non-transynaptic tract tracing experiments

4.2.1. Stereotaxic interventions

Experiment 1 (7 animals): To determine the projections of neurons of the PPN, the primarily anterograde tracer biotinylated dextrane-amine (BDA) (MW 10,000, Sigma-Aldrich, St. Louis, MO) was administered into this area using iontophoresis and labeled fibers were looked for in the medial basal hypothalamus.

Experiment 2 (5 animals): The primarily retrograde tracer FG (Fluorochrome Inc., Englewood, CO) was administered iontophoretically into the region of the ARC and labeled cell bodies were looked for in the peripeduncular region of the midbrain.

On the day of the surgery, animals were anesthetized using 4% chlorohydrate (1ml/100g) and placed into a stereotaxic instrument (Benchmark Digital Stereotaxic – myNeurolab, St. Louis, MO). A single midline scalp incision was made to visualize the surface of the skull. The points of bregma and lambda were leveled and a small window was cut in the skull to expose the brain at Paxinos-Watson (1986) atlas coordinates: PPN – A 4,2mm, V 3.10mm and L 3.50mm or ARC – A 6.20mm, V 0.25mm and L 0.15mm, to

the interaural line and the midline, respectively. The sagittal sinus was removed to expose the interhemispheric fissure, which was used as the reference midline. A glass micropipette (30μm tip diameter) filled with 10% BDA or 2% FG dissolved in 0.9% saline was used to apply the tracers using positive pulses of 2uA, alternating 5 seconds on and 5 seconds off for a total of 5 minutes. It was then removed and the scalp was closed with wound clips. Following the surgery, the animals were returned to the animal colony.

4.2.2. Perfusion and tissue sectioning

Ten to 14 days following surgery the animals were anesthetized with an overdose of sodium pentobarbital (100mg/kg), the blood was flushed out by physiological saline containing 2% sodium nitrite solution and perfused transcardially with 0.1M potassium-phosphate buffered saline (KPBS) containing 4% paraformaldehyde (PFA) solution and 2.5% acrolein (pH 6.8). An additional rinse of physiological saline was used to remove residual PFA and acrolein. Brains were immersed into a 30% sucrose solution and later brains were cut in coronal plain on a freezing sliding microtome at 25μm and collected into an ethylene-glycol containing cryoprotectant/anti-freeze solution (Watson et al 1986). Sections were stored at -20˚C until use.

4.2.3. Immunohistochemistry

4.2.3.1.Single Stains

Experiment 1 (visualization of BDA): Sections were removed from the cryoprotectant/anti-freeze solution, rinsed with KPBS several times, and then incubated in 1% sodium borohydride/KPBS for 20 minutes to remove residual aldehydes and acrolein. The sodium borohydride solution was rinsed out of the tissue with KPBS. The sections were incubated in goat anti-biotin (Vector Laboratories, Burlingame, CA) at a dilution of 1:70,000 made up in KPBS with 0.4% Triton X-100 for 48 hours at 4°C. Following primary incubation, sections were rinsed with KPBS and then immersed into donkey anti-goat biotinylated secondary antibody solution (Vector Laboratories, Burlingame, CA) at a dilution of 1:600 for an hour at room temperature. The tissue was then rinsed and placed into avidin-biotin complex solution (ABC Elite Kit, Vector Laboratories, Burlingame, CA) for an hour at room temperature, then rinsed with KPBS, followed by rinses with 0.175M sodium acetate solution. The BDA was visualized using a nickel sulfate - 3, 3-

diaminobenzidine tetrahydrochloride (DAB) chromogen with H_2O_2 in 0.175M sodium acetate. The reaction was stopped by rinses with sodium acetate. The specificity of BDA labeling was demonstrated by omiting primary and secondary antibodies using only an ABC Kit. This method also resulted in labeling; however, the application of anti-biotin antibody and biotinylated secondary antibody extremly enhanced the intensity of labeling. With the use of this technique, a high dilution of anti-biotin antibody (1:70,000) was effective. The above-mentioned technique was usually used in our laboratory.

Experiment 2 (visualization of FG): The staining procedure is similar to Experiment 1, but sections were incubated in rabbit FG antibody (Chemicon, Temecula, CA) at a dilution of 1:100,000 for 48 hours at 4°C, then in goat anti-rabbit biotinylated secondary antibody solution, then in ABC complex and nickel-DAB chromogen. The specificity of FG labeling was demonstrated omiting primary and secondary antibodies and ABC Kit. FG alone resulted in weak fluorescence signal. With the use of primary and secondary antibodies, ABC Kit and nickel intensified DAB chromogen the labeling was extremly enhanced.

Only those animals were included in the experiments, where the stereotaxic interventions were located in the targeted areas.

4.2.3.2. Double Stains

Experiment 1: To demonstrate the relation of BDA labeled fibers ascending from the injection site to TIDA and DYN neurons residing in the ARC, we conducted BDA and TH, and BDA and DYN double labeling. The visualization procedure of BDA has been described above. After completing the BDA stain, sections were rinsed and incubated with monoclonal TH antibody (Chemicon, Temecula, CA) at a dilution of 1:500,000 for 24 hours. The sections were rinsed with KPBS and then immersed into horse anti-mouse biotinylated secondary antibody solution (Vector Laboratories, Burlingame, CA) at 1:600 for an hour at room temperature. Following rinses, the tissue was placed into avidin-biotin complex solution (ABC Elite Kit, Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. TH was visualized using DAB chromogen with H_2O_2 in Tris buffer (pH 7.5) without nickel intensification. Another set of BDA stained sections was incubated with rabbit DYN antiserum (Peninsula

Laboratories, Belmont, CA) at a dilution of 1:20,000 for 24 hours, then with goat anti-rabbit biotinylated antiserum. After tyramide amplification (Tyramide Amplification Kit was purchased from New England Nuclear, PerkinElmers, Waltham, MA), the final reaction product was visualized with streptavidin Cy3 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). To demonstrate the relation of DYN and TIDA neurons, a series of sections containing ARC was stained for DYN and TH immunoreactivities using ABC technique and nickel intensified DAB chromogen for demonstrating DYN and only DAB chromogen to demonstrate TIDA neurons. DYN antibody was purchased from Peninsula Laboratories (Belmont, CA) and used at a dilution of 1:20,000 for 24 hours.

Experiment 2: To chemically characterize the FG containing neurons retrogradely labeled from the ARC, we stained the midbrain sections for CGRP, TIP39 or GAL immunoreactivity.

A. FG/CGRP double labeling. The vizualization of FG has been described above. Midbrain sections containing FG retrograde labeling were incubated in rabbit CGRP antiserum (Chemicon, Temecula, CA) at a dilution of 1:30,000 for 24 hours, then in goat biotinylated antibody at a dilution of 1:600 for an hour. The final reaction product was visualized by ABC complex and DAB chromogen only.

B. FG/TIP39 double labeling. FG and TIP39 were stained by double immunofluorescence staining. The sections were first incubated in rabbit FG antiserum, then in biotinylated secondary antibody, and finally in streptavidin Cy2 conjugate (dilution 1:500) for an hour. After rinsing, the sections were incubated in rabbit TIP39 antiserum raised and characterized by Usdin et al [1999] at a dilution of 1:15,000 for 48 hours. The sections were then incubated in goat anti-rabbit serum conjugated with Cy5 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at a dilution of 1:500 for 24 hours.

C. FG/GAL double labeling. The sections were stained for FG as described above. For visualization of FG, we have used nickel intensified DAB chromogen. The sections were then stained for GAL immunoreactivity using rabbit antiserum (Peninsula Laboratories

Inc., San Carlos, CA) at a dilution of 1:5,000. We used ABC technique, and DAB chromogen was applied without nickel intensification.

4.3. TH and ENK expression in TIDA neurons

Adult timed-pregnant rats were housed on a 12 hour light/12 hour dark light schedule (lights on at 3am and off at 3pm) with unlimited access to food and water. The females gave birth on gestation day 21 or 22 and pups were culled to 8 on post partum day (pp) 2. The dams were divided into 3 main groups with 5-6 rats investigated for each time point. Group I: Dams continued to suckle throughout the experiment.

Group II. Pups were removed on pp10 and the dams were perfused at 3-4, 6, 7-8, 10-12, 16-20, or 24-28 hours after removal.

Group III. Pups were removed on pp10 for 4 hours (the time at which previous studies indicated clear heteronuclear TH mRNA up-regulation) and then pups were returned for 3-4, 6-8, 12-16, or 20-24 hours. In the case of ENK study, based on initial patterns of change (which indicated a very slow decline in ENK expression) additional groups in which pups were removed for 48 and 72 hours were added.

Group IV. Females with diestrous II stage of estrous cycle were also included in the experiment in which ENK mRNA were determined.

To prepare the dams for mRNA analysis, dams were anesthetized with sodium pentobarbital (100mg/kg) and perfused transcardially with a solution of 0.9% sodium chloride with 2% sodium nitrite followed by 4% PFA solution containing 2.5% acrolein (pH 6.8). Brains were removed and transferred to a 30% sucrose solution. The brains were sectioned on a freezing sliding microtome at 25μm and collected into a cryoprotectant/anti-freeze solution and stored at –20˚C. This procedure enables collection of tissue over prolonged periods of time and then storage of the sections with full maintenance of mRNA levels for over 12 years with no decay (Hoffman and Le 2004).

4.3.1. In situ hybridization

4.3.1.1. Probe preparation

The pGEM3-TH3’ construct contains a 475 base pair EcoRI/HindIII fragment corresponding to amino acids 219-377 of the rat TH enzyme. This TH fragment was

derived from the RR1.2 plasmid obtained from Dr. D.M. Chikaraishi (Duke University). For antisense TH riboprobes (cRNA), the plasmid was linearized with HindIII and transcribed with T7 RNA polymerase, yielding a 509 nucleotide complementary RNA (cRNA).

A 693-bp rat ENK cDNA construct gift of Stanly Watson (University of Michigan and permission from Dr. Audrey Seasholtz) subcloned in SphI-SmaI site of pGEM3-3Z plasmid was linearized with HindIII and transcribed with T7 RNA polymerase to synthesize antisense proenkephalin cRNA probe *in vitro*. For sense probe, this plasmid was linearized with Ava1 and transcribed with SP6 RNA polymerase.

The *in vitro* transcription reaction mixture contained 1.0mM Biotin-16-uracil triphosphate (UTP) (Roche, Indianapolis, IN), 1μg HindIII-linearized-pGEM3z-TH3′, 4mM DTT, 40 units T7 RNA polymerase (Roche, Indianapolis, IN), 0.35mM UTP, and 1.0mM each of adenosine triphosphate (ATP), guanine triphosphate (GTP), and cytosine triphosphate (CTP). The transcription reaction was stopped by the addition of 1μl of ethylenediaminetetraacetic acid (EDTA). For an RNA sense probe, the pGEM3-TH3′ plasmid was linearized with EcoRI and transcribed with Sp6 RNA polymerase. The targeted sequences of nucleotides for the antisense probe are all contained within a single exon thus enabling the probe to bind to either mature or heteronuclear RNA in the tissue.

4.3.1.2. Hybridization and visualization

Day 1 (RNAse free). Sections were removed from the cryoprotectant/anti-freeze solution and rinsed KPBS made with 0.1% Diethyl Pyrocarbonate water (DEPC H_20), then incubated in 1% sodium borohydride/KPBS+DEPC H_2O to remove residual aldehydes and acrolein. Sections were then rinsed repeatedly. The tissue was rinsed with 0.1M triethanolamine buffer (TEA, pH 8.0) followed by an incubation in 0.25% acetic anhydride in TEA at room temperature. Sections were then washed with 2 x SSC (0.3M NaCl, 0.33M NaCitrate, pH 7.0) solution and prehybridized at 50°C for 2 hours by using prehybridization buffer (50% deionized formamide, 10% dextran sulfate, 1 x Denhardt's solution, 300mM NaCl, 8mM Tris pH 8.0, 0.8mM EDTA, 15% DEPC H_2O) containing 2mg/ml of heat denatured torula yeast RNA (TRNA) (Ambion, Austin, TX). After rinsing with 2 x SSC followed by hybridization with biotinylated probe (final

concentration of 600ng/kbp/ml), the probe and TRNA were heat denatured, mixed with hybridization buffer, placed on the tissue, and incubated overnight at 50°C.

Day 2. Tissue was rinsed with 4 x SSC for 30 minutes, once with RNAse buffer (10mM Tris pH 8.0, 500mM NaCl, 0.75mM EDTA, pH 8.0), heated to 37°C and incubated in RNAse (20ug/ml) in RNAse buffer at 37°C. Following rinses with RNAse buffer, the tissue was incubated in RNAse buffer at 37°C. After an hour of 2 x SSC, 1x SSCs, and 0.1 x SSC rinses, the tissue was incubated in 0.1 x SSC for 60 minutes at 55°C. After incubation the tissue was rinsed with KPBS, then incubated in goat anti-biotin (Vector Laboratories, Burlingame, CA) at a concentration of 1:100,000 in KPBS+0.4% Triton X-100 at 4°C for 48 hours.

Day 4: Following incubation of the sections with primary antiserum, sections were rinsed with KPBS and then immersed into donkey anti-goat secondary antiserum solution (Vector Laboratories, Burlingame, CA) at 1:600 in KPBS with 0.4% Triton X-100 at room temperature for 1 hour. The tissue was then placed into avidin-biotin complex solution (ABC Elite Kit, Vector Laboratories, Burlingame, CA). Following rinses with KPBS and 0.175M sodium acetate solution, the TH or ENK mRNA was visualized using a nickel sulfate 3, 3 DAB chromogen with H_2O_2 in 0.175M sodium acetate. The reaction was stopped by rinsing with the sodium acetate solution followed by rinses with KPBS. The sections were then placed into saline and mounted onto gelatin-subbed slides and later coverslipped with Histomount (National Diagnostics, Atlanta, GA).

4.3.2. Immunocytochemistry

Day 1: Sections were removed from the cryoprotectant/anti-freeze solution, rinsed with KPBS several times, and then incubated in 1% sodium borohydride/KPBS for 20 minutes to remove residual aldehydes and acrolein. Sections were then rinsed to remove the sodium borohydride solution and were incubated for 48 hours in rabbit anti-ENK (Incstar, Stillwater, MN) at a concentration of 1:300, made up in KPBS with 0.4% Triton X-100.

Day 3: Following incubation in the primary antiserum, sections were rinsed with KPBS and then immersed into donkey anti-rabbit secondary antibody solution (Vector

Laboratories, Burlingame, CA) at 1:800. The tissue was then rinsed and placed into avidin-biotin complex solution (ABC Elite Kit, Vector Laboratories, Burlingame, CA), then rinsed with KPBS, followed by rinses with 0.175M sodium acetate solution. The reaction product was visualized using a nickel sulfate 3, 3-DAB chromogen with H_2O_2 in 0.175M sodium acetate. The reaction was stopped by sodium acetate.

4.3.3. Image Analysis of the mRNA

Three sections containing representative areas of the ARC were included in the analysis. The slides were coded so the observer was blind to the animal's treatment. The sections were placed under a Nikon Eclipse 800 microscope linked to a Cooke camera and two levels of the ARC were examined, with two images captured at 60x on the left side of the third ventricle. IP Spectrum Software (Vienna, VA) installed on a Macintosh G4 computer was used for capturing and analyzing the images. The entire thickness of the sections was photographed in the Z plane in 0.2μm intervals. Only the 10 middle frames were then collapsed into a flattened image to visualize all mRNA clusters present within a 2μm thickness of a section. To determine the optical density (OD) of the mRNA grains, the OD of the background was subtracted from each image. Each cell was outlined as the regions of interest, segmented, and the OD for each cell containing TH or ENK mRNA determined separately. Values were expressed as means ± SEM for each experimental group. Differences in the mean grey levels between pup returned, removed but not returned and control groups were determined by One Way Analysis of Variance. the Tukey-Kramer post hoc comparison analysis for all pairs was performed, $p<0.05$ was considered statistically significant.

4.3.4. Image Analysis of the ENK peptide immunoreactivity in the ME

Three sections of the ME were analyzed per animal. The slides were coded so the observer was blind to the animal's treatment. The sections were placed under a Nikon Eclipse 800 microscope linked to a Cooke camera. IP Spectrum Software (Vienna, VA) installed on a Macintosh G4 computer was used for capturing and analyzing the images. The images were captured at 20x. The captured images were then normalized and segmented. The region of interest, namely the ME, was outlined using a draw tool. The segmented area and the region of interest were then quantified. ENK

immunohistochemistry was analyzed using One Way Analysis of Variance, complemented with the Tukey-Kramer post hoc comparison analysis for all pairs.

4.4. Transynaptic tract tracing experiments

Four Wistar (W) and nine Sprague Dawley (S-D) female primiparous (2-3 month old) rats between 7 and 15 postpartum days were used for the experiments. After delivery the number of litters was reduced to eight. They were kept in a light (14 h light and 10 h dark schedule, light on from 7 am till 7 pm) and temperature controlled (maintained at 22±2°C) vivarium. The interventions were carried out under general anesthesia by ketamine-hydrochloride (75mg/100g bw) (Sigma, St. Louis, MO).

The dams were inoculated with a virus labeled with green fluorescence protein (GFP) in 2µl physiological saline ($8x10^8$ plaque forming unit). The virus was injected in the first and second nipple areas at right side. The animals were sacrificed at different times (2-4 days) after the inoculation.

4.4.1. Preparation of virus

A genetically modified pseudorabies (PRV) strain termed memGreen-PRV was used for the tracing experiments. The consruction of memGreen-PRV was described by Boldogkői and his coworkers (2000). The wild type strain Kaplan of PRV was modified by elimination the gE and gI genes of the virus, which resulted in a viral tracer spreading in an esclusively retrograde direction. The gE and gI genes were replaced by a gene expression cassette encoding a membrane-bound green fluorescence protein which makes easy the identification of the virally-infected cells.

4.4.2. Tissue preparation

All animals were again anesthetized at the end of the experimental period and the blood was flushed out through the ascending aorta, then the animals were perfused by 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) in potassium phosphate buffer (KPB) (0.1M, pH 7.4). The components were purchased from Sigma-Aldrich (St. Louis, MO). After 24 hour postfixation the right 2nd nipple area with the underlying mammary tissue, the right sympathetic trunk, the upper thoracic segments of the spinal cord and the brain were blocked and placed in ascending sucrose solution (10-20-30%), then

DOI:10.14753/SE.2012.1755

embedded in Cryomatrix (Thermo Shandon, Pittsburg, PA). Twenty μm thick sections were cut on cryostate (Cryotome, Thermo Shandon, Pittsburg, PA). In all parts of the nervous system, what we took out, GFP labeling was looked for to demonstrate the presence of retrogradely transported virus. Sympathetic trunk, the spinal cord, medulla oblongata and the hypothalamus were immunostained for both dopamine-β-hydroxylase (DBH) and vesicular acetylcholin transporter (VAChT). The hypothalamus was also stained for OXY. The mammary tissues were stained for S-100 protein, CGRP, DBH and VAChT immunoreactivities.

4.4.3. Immunohistochemistry

After washing, the slides were treated with 1% Triton X-100 for better penetration of antibodies. The following primary antisera were used for immunostaining against: *1)* S-100 (a protein present in the peripheral nerve sheath), raised in rabbit and purchased from DAKO Co. (Carpinteria, CA) in a 1:1000 dilution. *2)* VAChT (present in cholinergic nerve fibers) raised in guinea pig and purchased from Milipore (Boston, MA) in a 1:500 dilution. *3)* DBH (an enzyme present in adrenergic and noradrenergic nerve fibers) raised in mouse and purchased from Milipore (Boston, MA) in a 1:1000 dilution. *4)* CGRP raised in rabbit by Görcs and characterized by Jakab and his coworkers (1990) in 1:2000 dilution. Antigen-antibody complex was visualized using indirect immunofluorescence technique. Fluorescence labeled secondary antibodies against rabbit, guinea pig and mouse immunoglobulins were purchased from Sigma and applied in a dilution 1:400.

The specificity test: Omitting the primary antibodies prevented the immunostaining. We also used positive controls such as the brain stem in the case of DBH and S-100 and anterior horn of the spinal cord in the case of VAChT, where the positive staining was well established.

DOI:10.14753/SE.2012.1755

5. RESULTS

5.1. Morphological studies of the SIPR pathway

5.1.1. Experiment 1: Anterograde tracer (BDA) injection in the rostral mesencephalon

Connection between the mesencephalon and ARC

BDA administered exclusively into the PPN failed to label any fibers inside the ARC; however, labeled fibers were found in the vicinity of the ipsilateral ARC and the ventromedial hypothalamic (VMN) nuclei (not shown). Administration of the tracer ventral and medial to the PPN actually did label fibers in the ipsilateral ARC. According to the stereotaxic rat brain atlas (Paxinos and Watson, 1986) this mesencephalic area was identified as the parvocellular part of the subparafascicular nucleus (SPFpc). Fig. 12A and B shows the PPN and SPFpc in the cross-section of the mesencephalon at A4,2 to the interaural line and the localization of the injection site of BDA in the mesencephalon. From the injection site BDA was anterogradely transported into the ARC where we have observed BDA labeled fibers (Fig. 12C). BDA was demonstrated by immunohistochemistry.

The neurons in the proximity of BDA labeled fibers in the ARC did not seem to be TIDA neurons, the fibers were mostly located lateral to these cells (Fig. 13). The BDA/TH double label immunostaining proved our observation correct. BDA labeled axons did not contact TH containing TIDA neurons in either case. An example is shown by the insert of Fig. 13.

Interaction between BDA fibers of mesencephalic origin and DYN neurons

When the hypothalamic slides containing BDA fibers were stained for DYN immunoreactivity, it was realized that BDA fibers were close apposition on DYN immunoreactive cell bodies which are located in the ventrolateral part of ARC (Fig. 14).

DOI:10.14753/SE.2012.1755

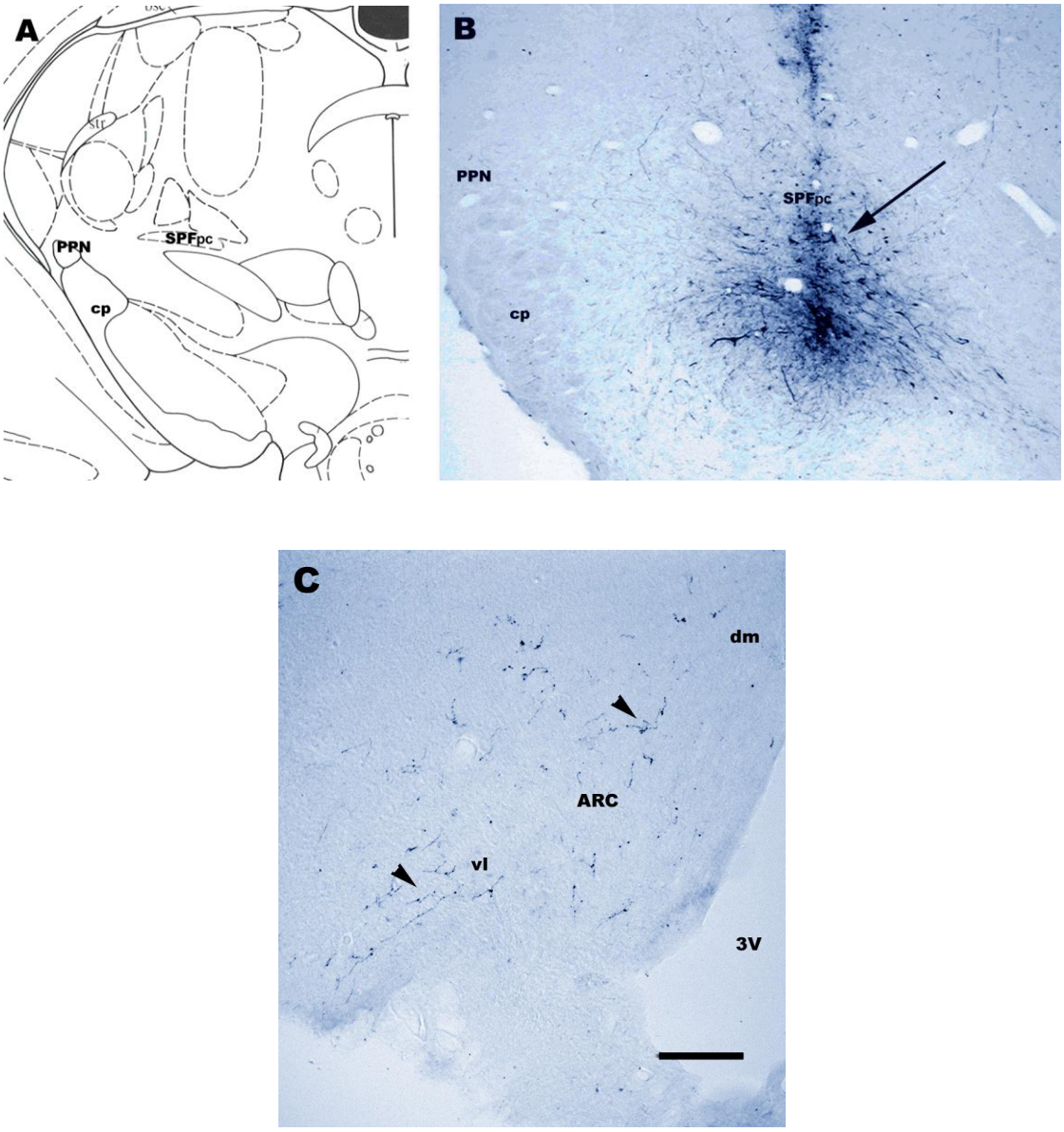

Fig. 12. Site of BDA injection in the mesencephalon is shown by A and B. C shows BDA fibers in the ipsilateral ARC**. A**. Schematic illustration of trans-section of the mesencephalon according to Paxinos and Watson (1986) at A4,2 to interaural line**. B.** Microphotograph shows the trans-section of mesencephalon with the injection site of BDA at the same level as A. Injection site includes SPFpc. **C**. Microphotograph shows BDA labeled fibers in ARC (frontal section of the medial basal hypothalamus). BDA was anterogradely transported to ARC from the mesencephalic site of BDA injection (B). Arrow shows the injection site of BDA (B), arrowheads show BDA labeled fibers (C). Abbreviations: 3V = third ventricle; ARC = arcuate nucleus; cp = cerebral peduncle; dm = dorsomedial part of ARC; PPN = peripeduncular nucleus; SPFpc = subparafascicular nucleus, parvocellular part; vl = ventrolateral part of ARC. Scale: 125μm in B and C.

DOI:10.14753/SE.2012.1755

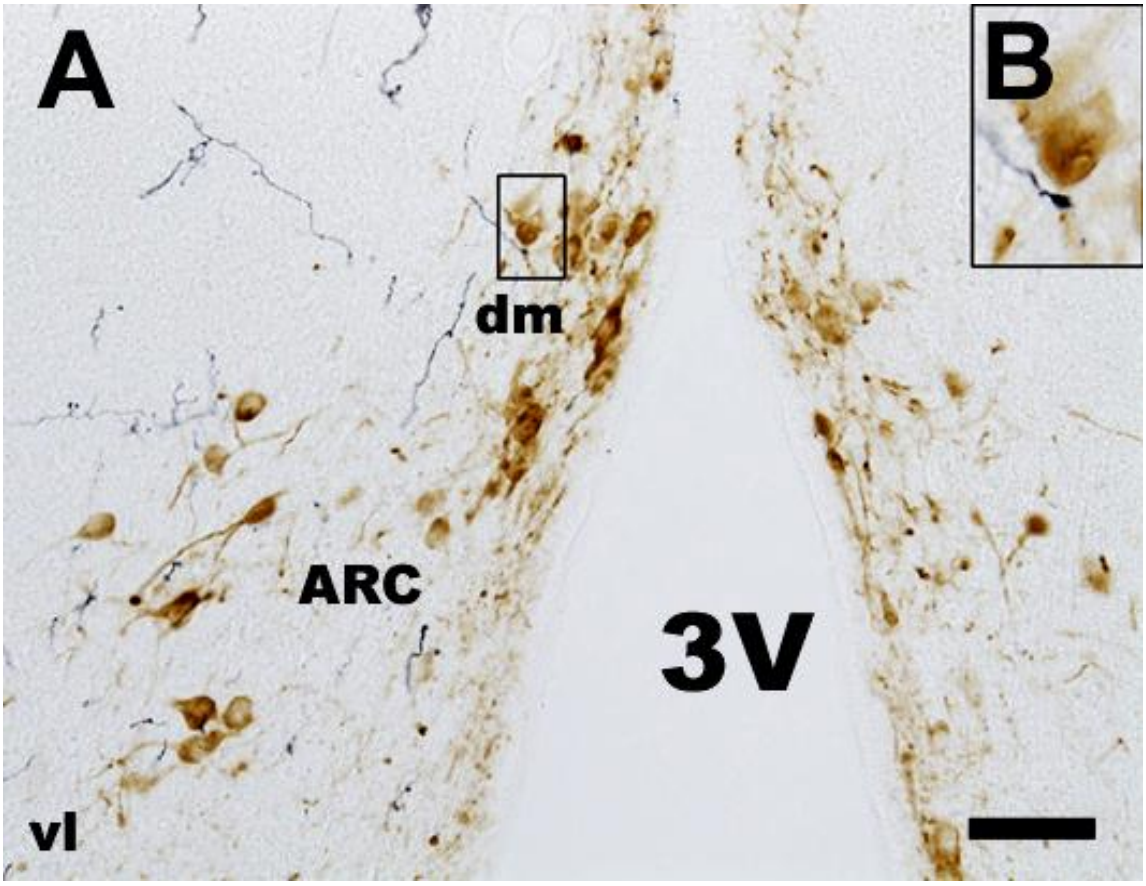

Fig. 13. BDA and TH double labeling in the frontal section of the medial basal hypothalamus (**A**). Insert (**B**) shows a high power detail of ARC (in rectangle) ipsilateral to the site of BDA injection in the mesencephalic SPFpc. TH immunopositive cells (indicated by brown colour) in the dorsomedial part of ARC do not show any contact with BDA labeled fibers (indicated by blue colour). Abbreviations: 3V = third ventricle; ARC = arcuate nucleus; dm = dorsomedial part of ARC; vl = ventrolateral part of ARC. Scale: 100μm in A and 25μm in B.

DOI:10.14753/SE.2012.1755

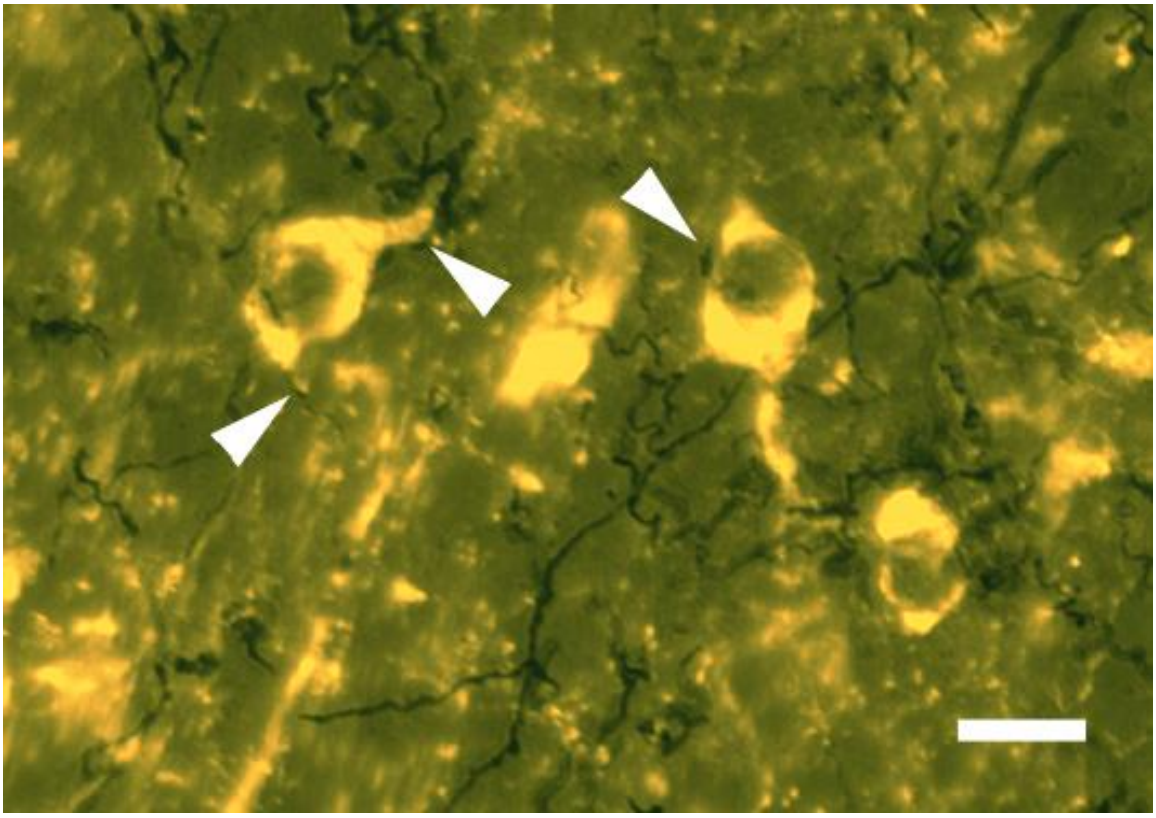

Fig. 14. Microphotograph shows DYN immunoreactive cell bodies (orange colour) and BDA fibers (black colour). BDA fibers exhibit close apposition on DYN immunoreactive cell bodies and dendrites (indicated by white arrowheads). Scale: 25μm.

DOI:10.14753/SE.2012.1755

In turn, DYN fibers innervate TIDA neurons which are located in the dorsomedial part of ARC (Fig. 15).

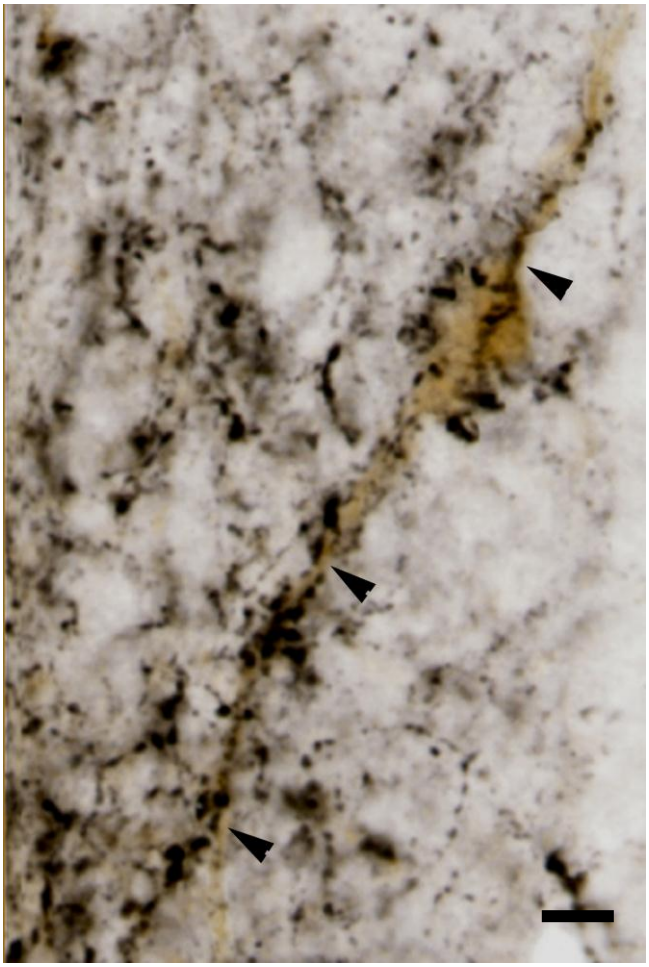

Fig. 15. Microphotograph shows a TIDA neuron (brown colour) which is heavely innervated by DYN fibers (black colour). Arrowheads show close apposition of DYN fibers and dendrites and cell bodies of TIDA neurons. Scale: 20μm.

5.1.2. Experiment 2. Retrograde tracer (FG) injection in the lateral ARC

In the midbrain of those animals where FG administration included the antero-ventro-lateral portion of the ARC (Fig. 16A) there was FG labeling in an area located in the rostral mesencephalon that consisted of horizontally oriented cells (Fig. 16B) extending from rostromedial to caudolateral direction fusing with the posterior intralaminal nucleus and PPN. This region is SPFpc.

DOI:10.14753/SE.2012.1755

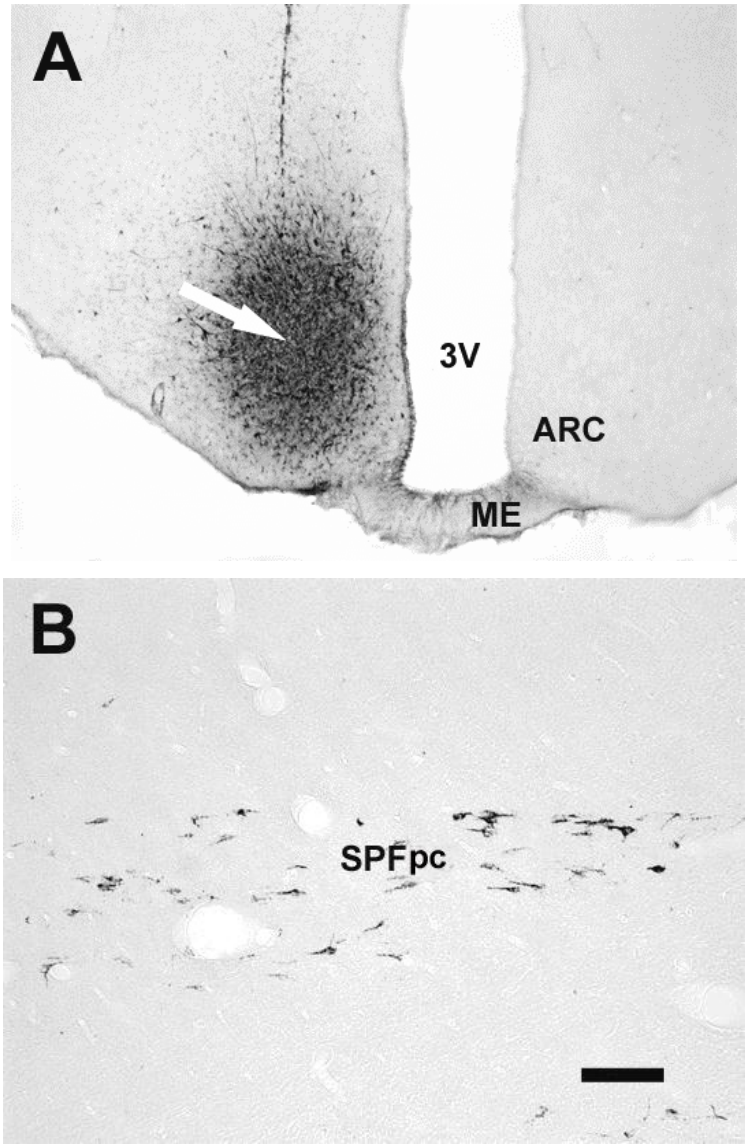

Fig. 16. **A**. The site of FG injection in the ARC (A6,2 to interaural line). **B**. FG labeled cells in the mesencephalic SPFpc. The site of injection includes the ventrolateral part of ARC indicated by white arrow. Abbreviations: 3V = third ventricle; ARC = arcuate nucleus; ME = median eminence; SPFpc = parvocellular part of subparafascicular nucleus. Scale: 250μm in A and 50μm in B.

DOI:10.14753/SE.2012.1755

A. FG and CGRP double labeling suggests that the subpopulation of the cells that projects to the ARC from the SPFpc also contains CGRP (Fig. 17).

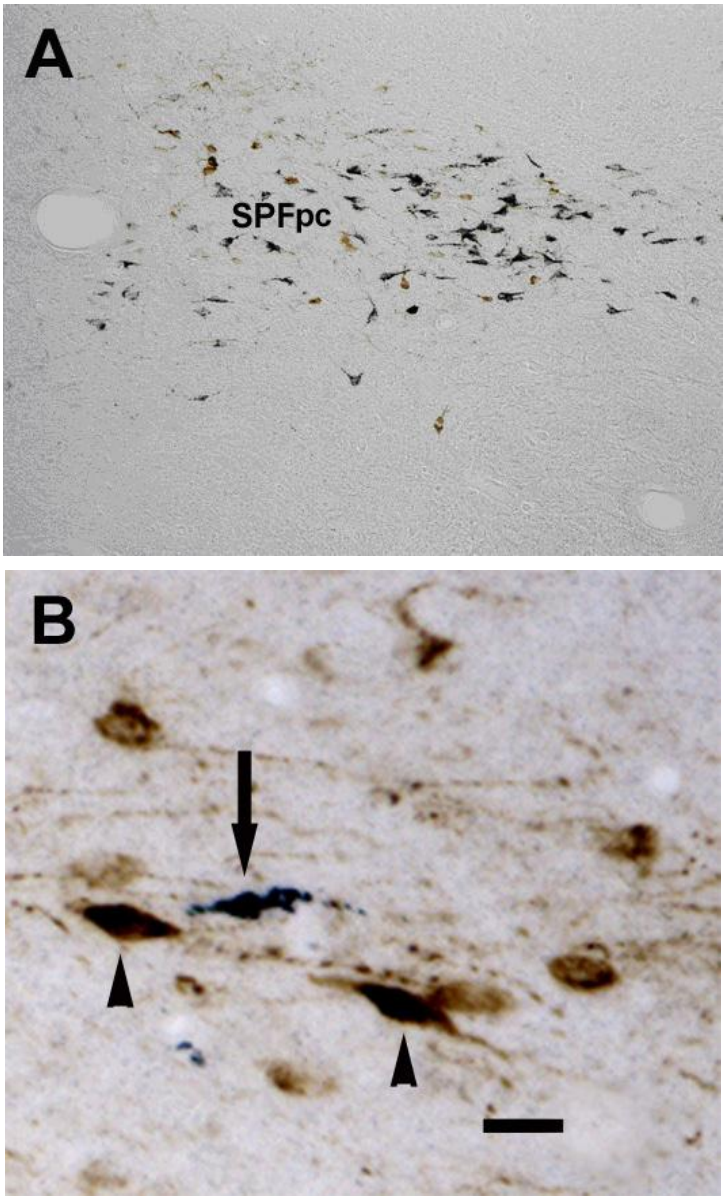

Fig. 17. **A**. Microphotograph shows retrograde FG labeling in SPFpc after FG injection into ARC (bluish black colour). The sections were also stained for CGRP immunoreactivity (brown colour). **B**. High power detail of SPFpc. Arrow shows a cell labeled only with FG, arrowheads show CGRP immunoreactive cells that also contain FG. Abbreviation: SPFpc = parvocellular part of subparafascicular nucleus. Scale: 250μm in A and 25μm in B.

DOI:10.14753/SE.2012.1755

B. FG injection into the ARC, which included its ventrolateral region, also labeled some cells in the SPFpc which were TIP39 immunopositive (Fig. 18).

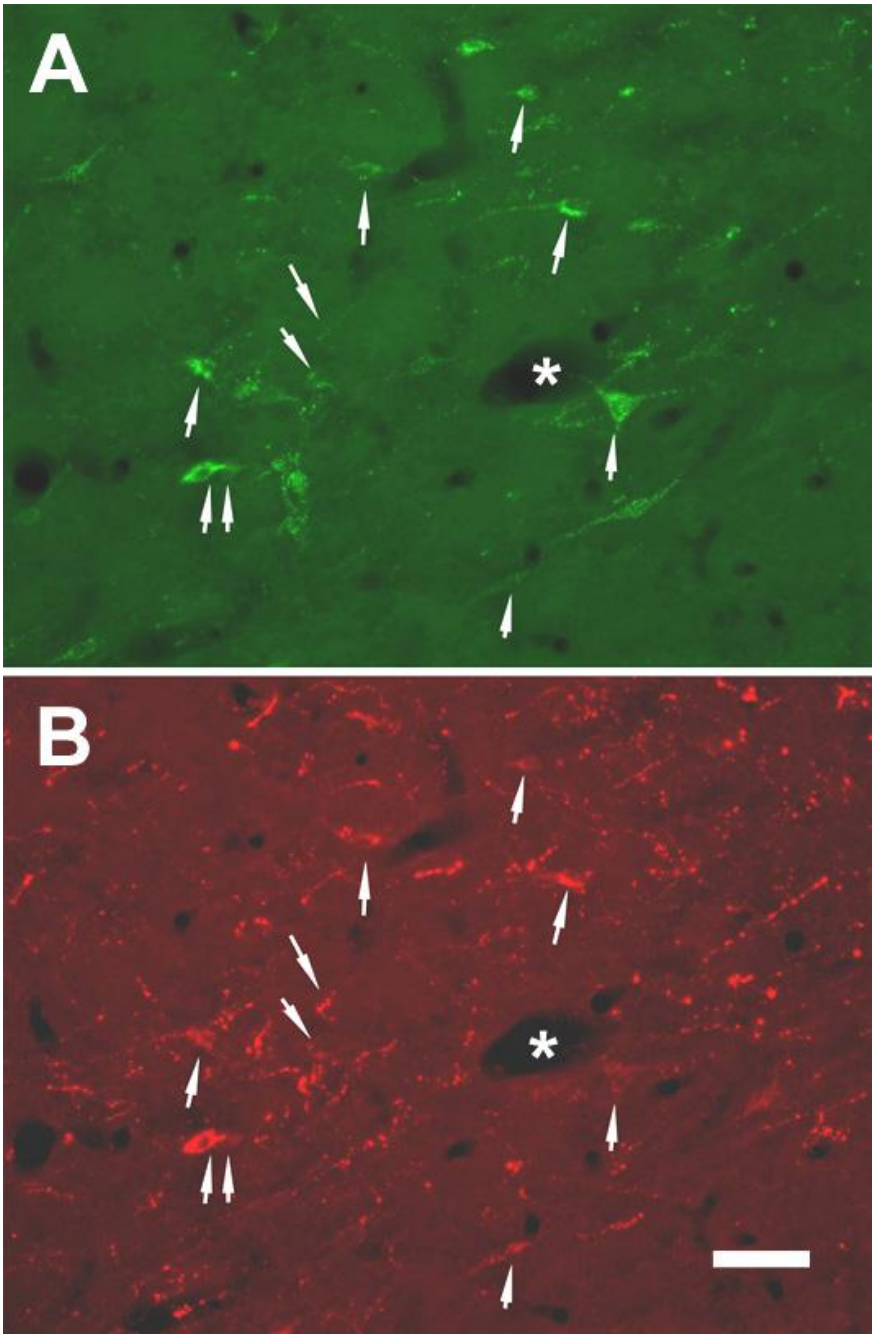

Fig. 18. FG injected in ARC labeled cells in the mesencephalic SPFpc. A part of the FG labeled cells (**A,** green fluorescence) also showed TIP39 immunoreactivity (**B,** red fluorescence). Arrows in the same position in A and B label the same cells. * indicates the same vessel in A and B. Scale: 50μm.

DOI:10.14753/SE.2012.1755

C. FG injection into the ARC did not label cells in the SPFpc which contained GAL (not shown).

Table 1 summarizes the results obtained by the antero- and retrograde tracing studies which were confirmed by double staining technique.

Table 1. Summary of the ante- and retrograde labeling.

Site of injection of tracers	**Site of Midbrain**		**transported tracer ARC**	
	PPN	SPFpc	TIDA region	Ventro-lateral region
FG - ARC	-	+		
BDA - PPN			-	-
BDA-SPFpc			-	+

5.2. Physiological studies

5.2.1. Quantitative analysis of TH mRNA in ARC

The analysis of TH mRNA expression by measuring OD (Fig. 19) revealed distinctive changes between rats that did not get their pups returned and rats whose pups resumed suckling after a 4 hour separation. TH mRNA levels were significantly higher by 6 hours in the group that did not get pups back than in continuously suckling controls. TH mRNA levels continued to rise after the complete termination of suckling and remained high even 28 hrs after pup removal. On the other hand, if the pups were returned after a 4 hour separation, the mean values of TH mRNA levels remained higher than in continuously suckling controls even 20-24 hrs after the resumption of suckling. By 16-20 hours after initial separation the dams whose pups were returned had significantly lower TH mRNA levels than those whose pups were removed but not returned. This indicates that returning the pups and thus the re-initiation of suckling started to suppress the TH mRNA production

DOI:10.14753/SE.2012.1755

in TIDA neurons, although the already up-regulated levels did not return to continuously suckling levels.

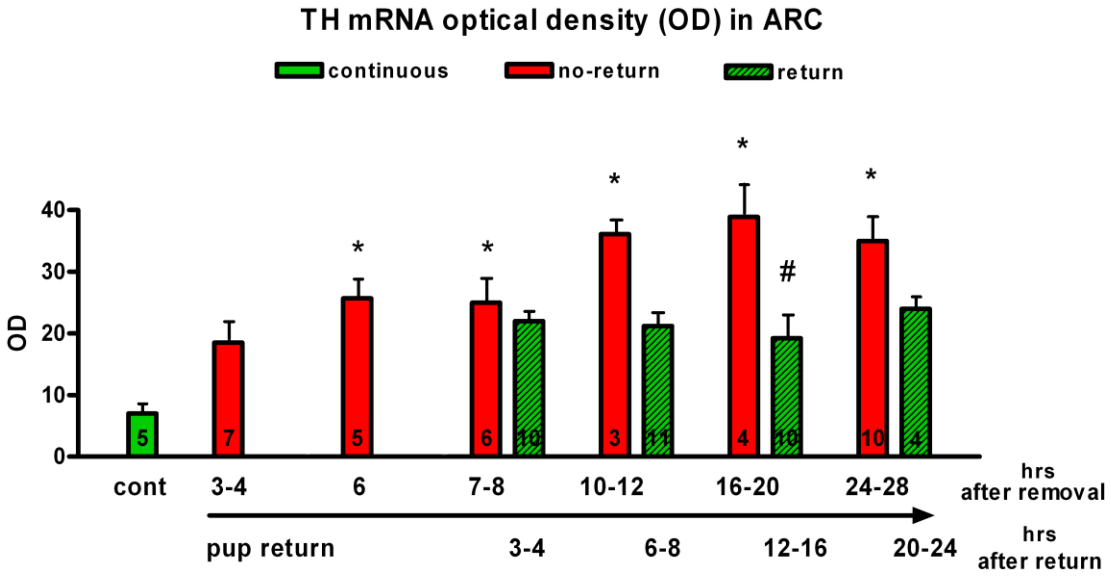

Fig. 19. Optical density (OD) of TH mRNA clusters in TIDA neurons of A12 region of ARC at different times after pup removal. The 'no-return' bars demonstrate the OD levels at different times after pups have been removed (and never returned). The 'return' bars demonstrate the OD levels at certain time points after pups were removed; however, these pups were returned after a 4 hour separation and continued suckling afterwards. The green bar represents a control group, in which the dams continued to lactate throughout the experiment without their pups being removed at all. The numbers in the bars indicate the number of rats used in each group. * indicates significant difference between continuously lactating group and the given 'no-return' group ($p<0.05$). # indicates significant difference between 'return' group and the corresponding 'no-return' group ($p<0.05$).

DOI:10.14753/SE.2012.1755

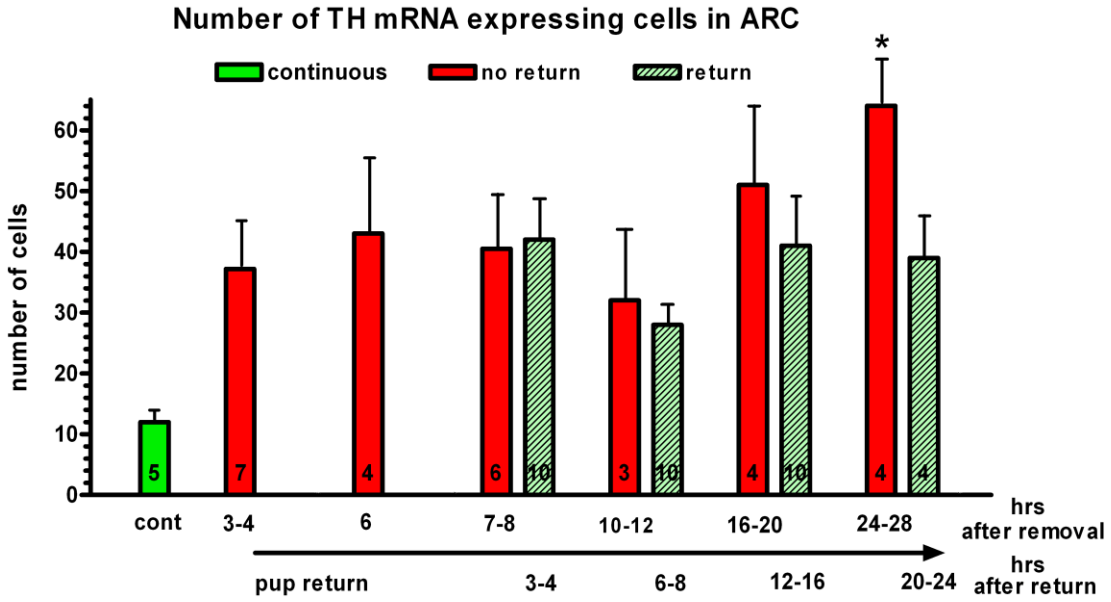

Fig. 20. The graph shows the number of cells detected in the A12 region of ARC that contain TH mRNA. The bars in different colours indicate similar groups as in Fig. 19. * indicates significant difference between continuously lactating group and the given 'no-return' group ($p<0.05$).

It was also examined how many cells containing TH mRNA clusters could be actually detected in ARC, regardless of the intensity of the mRNA expression (Fig. 20). The only significant difference found in the number of cells was between the continuously lactating group and the 24-28 hour pup-removed group.

5.2.2. Quantitative analysis of ENK mRNA in ARC

The analysis of ENK mRNA expression by measuring OD (Fig. 21) showed that expression of mRNA rose quickly after the termination of the suckling stimulus and was significantly higher than the OD in continuously suckling dams by 6 hours after pup-removal. After reaching peak levels around 7-8 hours, the levels declined and approached the continuously suckling levels. Return of the pups and thus resumption of the suckling stimulus after a 4 hour pup separation still resulted in an increasing trend in the means of expressed mRNA, although the levels were not significantly higher than those in

DOI:10.14753/SE.2012.1755

continuously suckling dams. Unexpectedly, the OD in continuously suckling dams was not significantly higher than in cycling female rats during the diestrous II stage.

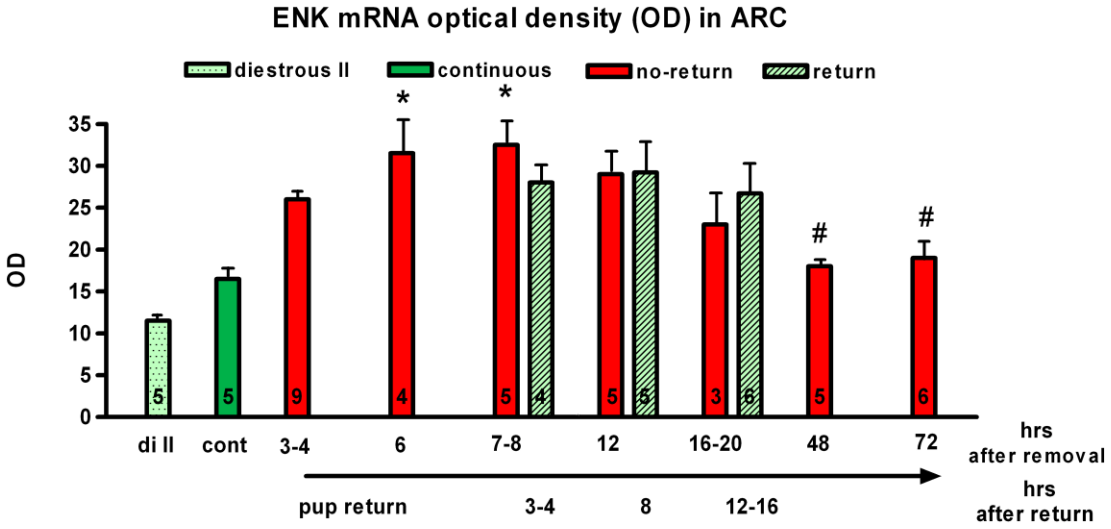

Fig. 21. The graph demonstrates the optical density (OD) of ENK mRNA clusters in the A12 region of ARC at different times after pup removal. The bars in different colours indicate similar groups as in Fig. 19. The dotted bar represents levels of cycling female rats at diestrus II. The numbers in the bars indicate the number of rats included in the group. * indicates significant difference between continuously lactating group and the given 'no-return' group ($p<0.05$). # indicates significant difference between 'no-return' group (7-8 hours) and 'no-return' groups (48 and 72 hours) ($p<0.05$).

The analysis of the number of ENK mRNA positive cells in the area of TIDA neurons in continuously suckling dams and pup removed dams did reveal significant changes at 7-8 hours after the cessation of suckling. There was a peak at that time and the number of positive cells gradually declined thereafter (Fig. 22). When the pups were returned the number of ENK expressing cells did not significantly differ from that of continuously lactating dams.

DOI:10.14753/SE.2012.1755

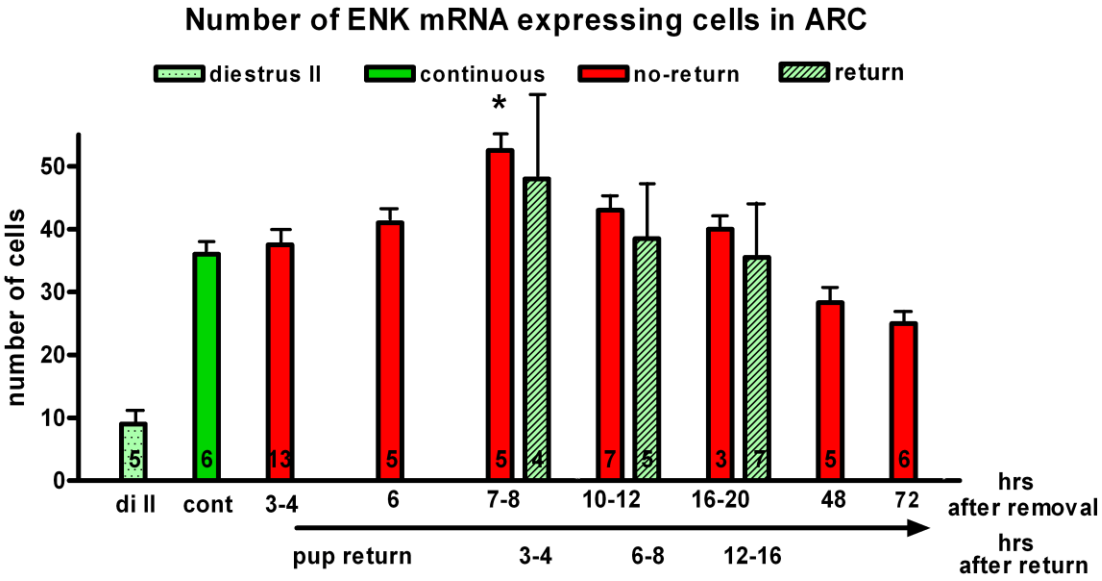

Fig. 22. The graph shows the number of cells detected in the A12 region of ARC that contain ENK mRNA. The red 'no-return' bars demonstrate the mean of the number of cells of each group at certain times after pups have been removed and never returned. The lined green 'return' bars demonstrate the means of the number of cells at certain time points after pups were removed; however, these pups were returned after a 4 hour separation and continued suckling afterwards. The green bar is a control group, in which the dams continued to lactate throughout the experiment without their pups being removed at all. The dotted green bar represents cycling female group in stage diestrus II. The numbers in the bars indicate the number of rats included in the group. * indicates significant difference between continuously lactating group and the given 'no-return' group ($p<0.05$).

5.2.3. Histological appearance of TH and ENK mRNA expressing cells in ARC

The histology of TH and ENK mRNA expressions well supports the qantitative data. In the dorsomedial part of ARC of a continuously lactating dam only a few TH expressing cells were observed (Fig. 23). The density of the reaction product was very low. Twenty four hours after removal of the pups the TH mRNA expression was extremly enhanced (Fig. 24).

DOI:10.14753/SE.2012.1755

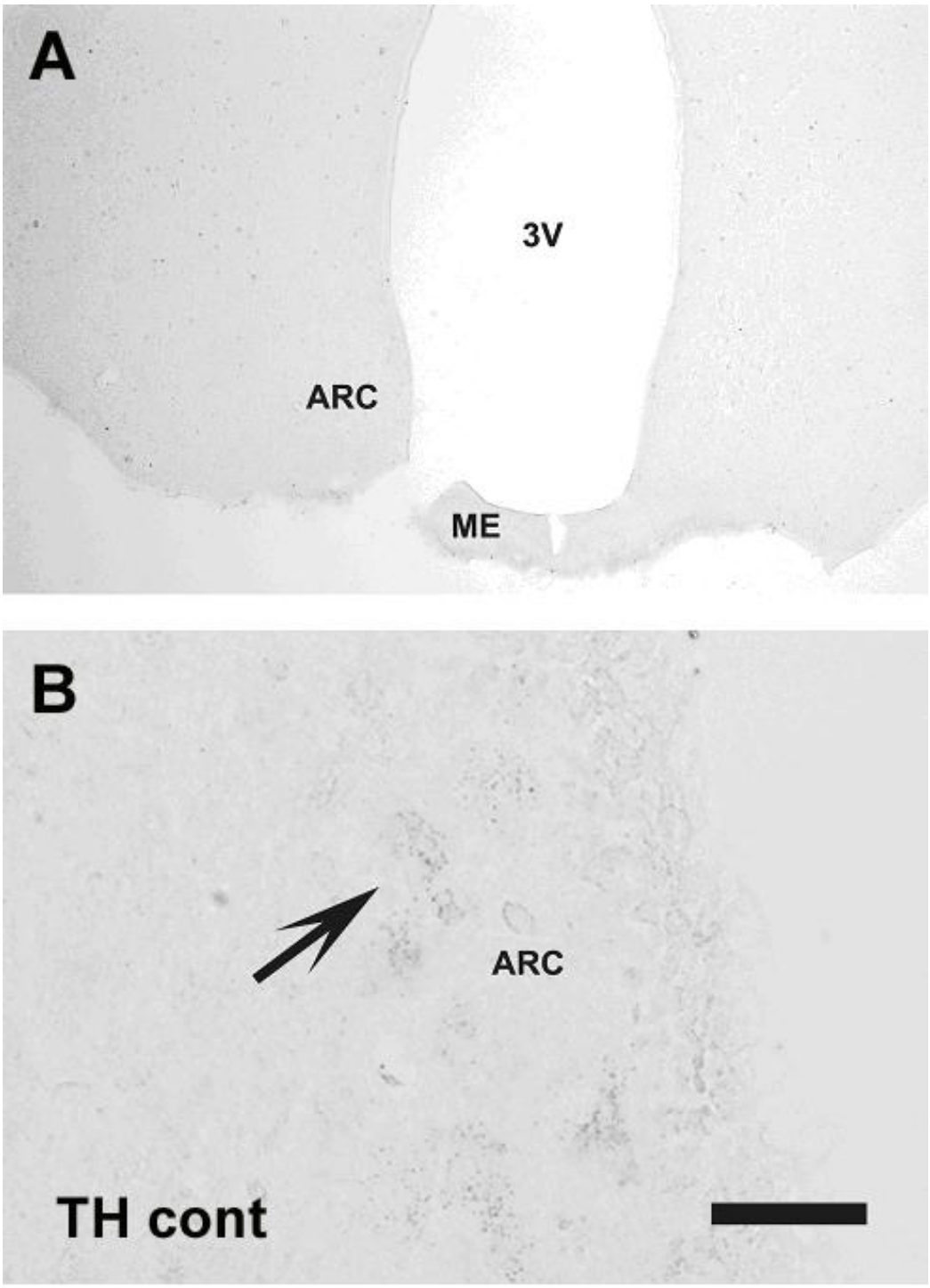

Fig. 23. Microphotographs show very moderate TH mRNA expression in a continuously lactating dam (**A** and **B**). Arrow shows TH expressing cells in TIDA region of ARC (B) which can only be recognized in a high power detail of ARC. Abbreviations: 3V = third ventricle; ARC = arcuate nucleus; ME = median eminence. Scale: 250μm in A and 50μm in B.

DOI:10.14753/SE.2012.1755

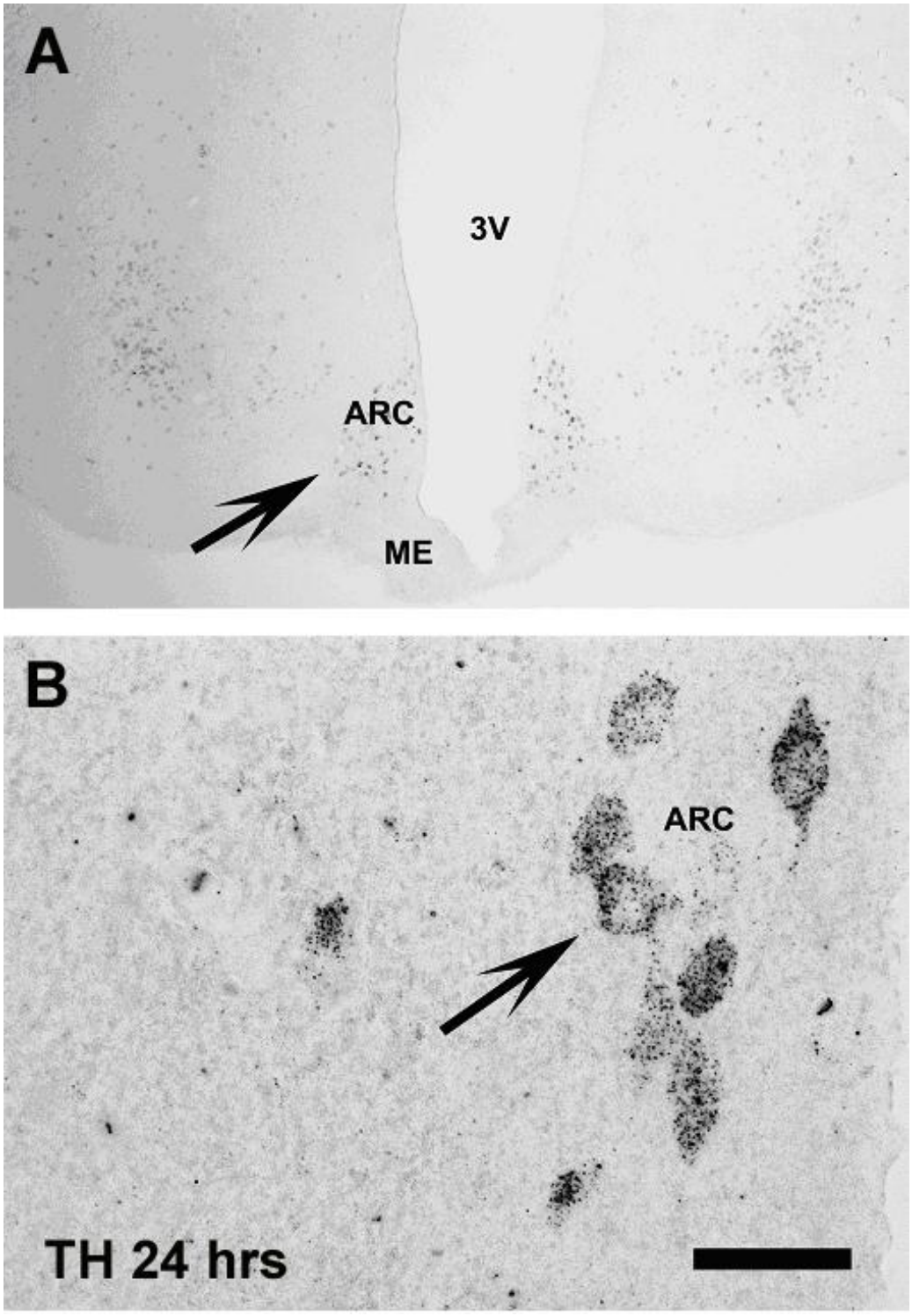

Fig. 24. Microphotographs show a strong TH expression in a dam whose pups were removed 24 hours earlier. Arrows show TH expressing cells in TIDA region of ARC (**A** and **B**). Abbreviations: 3V = third ventricle; ARC = arcuate nucleus; ME = median eminence. Scale: 250μm in A and 50μm in B.

DOI:10.14753/SE.2012.1755

The ENK mRNA expression was very low in diestrous rats (not shown), but extremly enhanced in continuously lactating rats (Fig. 25) and it was even stronger in the ARC of rats 8 hours after removal of pups (Fig. 26).

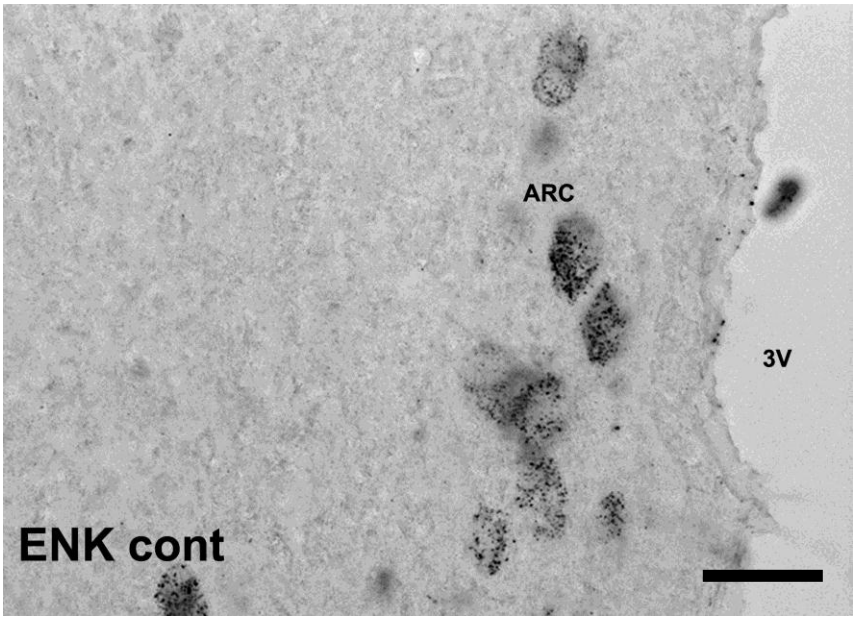

Fig. 25. ENK mRNA in the ARC nucleus of continuously lactating rats. ARC = arcuate nucleus; 3V = third ventricle. Scale: 50μm.

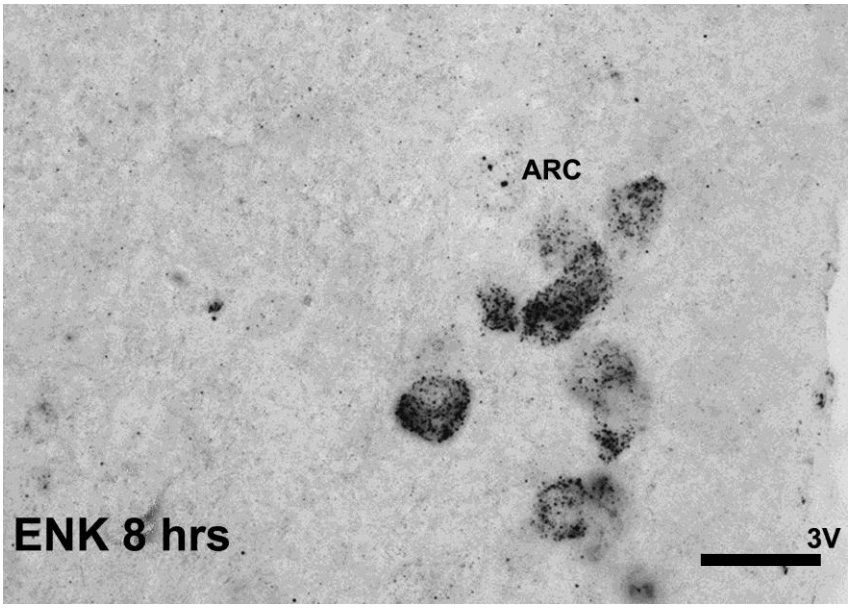

Fig. 26. ENK mRNA in the lactating rats 8 hrs after removal of pups. ARC = arcuate nucleus; 3V = third ventricle. Scale: 50μm.

DOI:10.14753/SE.2012.1755

5.2.4. Quantitative analysis and histological appearance of ENK peptide in the ME

The ENK immunostaining in the ME revealed a low ENK peptide level in cycling diestrous rats and a dramatically elevated level in continuously lactating dams as it is indicated by the difference in OD (Fig. 27) and by the histological appearance (Fig. 28 and 29). The elevation of OD is statistically significant compared to diestrous rats. 3-4 hours after the removal of pups, the peptide content of the ME dropped and continued to drop nearing diestrous levels by the end of the experimental period. The decrease became significant about 16 hours later (Fig. 27). In this case the histology of immunostaining also well supported the result of quantitative analysis (Fig. 30).

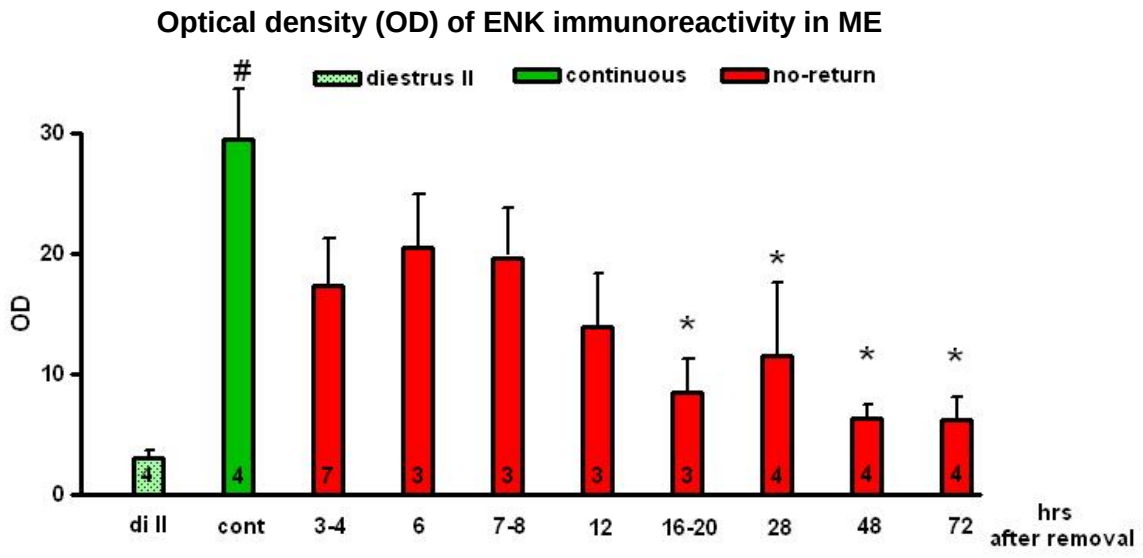

Fig. 27. The graph demonstrates the changes in the optical density (OD) of ENK immunoreactivity in the ME at different times following pup removal during lactation. The bar labeled "cont" represents the control group, where dams continued to lactate undisturbed throughout the duration of the experiment (10 days). The "di II" group is a group of cycling female rats euthanised during diestrus II of the ovarian cycle. The numbers above the bars indicate the number of rats used in the group. # = p<0,001 di II v. cont; * = p<0.05 cont v. no-return groups.

DOI:10.14753/SE.2012.1755

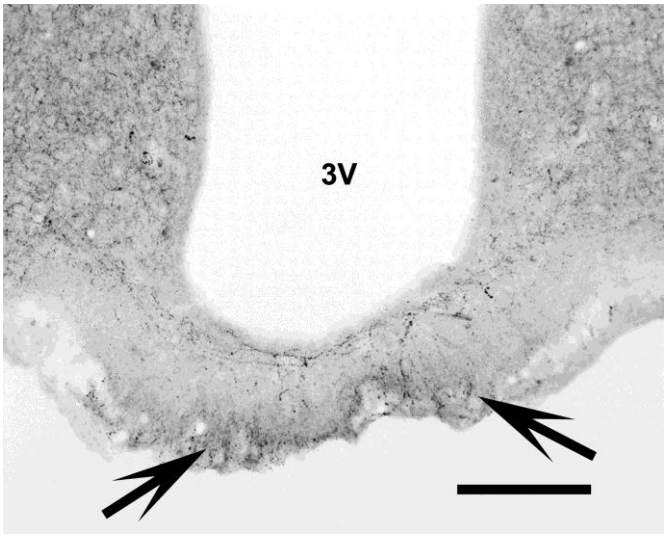

Fig. 28. Microphotograph shows ENK immunoreactivity in the ME of a diestrous female rat. Arrows indicate immunoreactive fibers. Abbreviation: 3V = third ventricle Scale: 250μm.

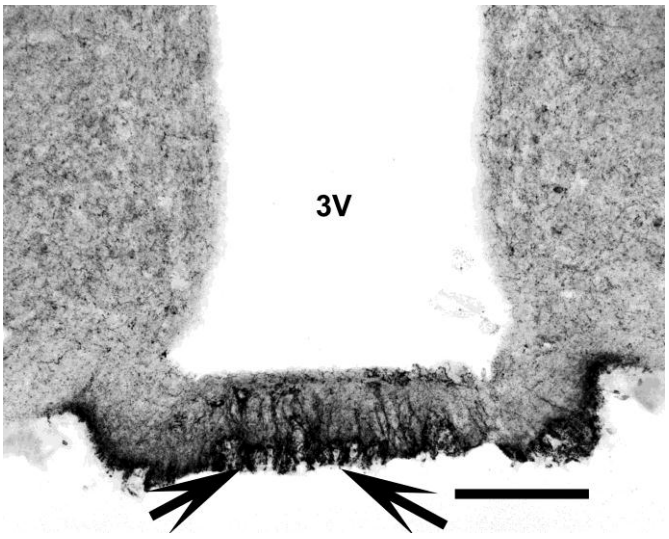

Fig. 29. Microphotograph shows ENK immunoreactivity in the ME of a continuously lactating rat. Arrows indicate immunoreactive fibers. Abbreviation: 3V = third ventricle. Scale: 250μm.

When we examined the magnitude of changes in the ENK protein in the ME it was found that in continuously suckling dams the magnitude was about 9 fold higher than in cycling diestrous rats and after pup removal it declined gradually (Fig. 31).

DOI:10.14753/SE.2012.1755

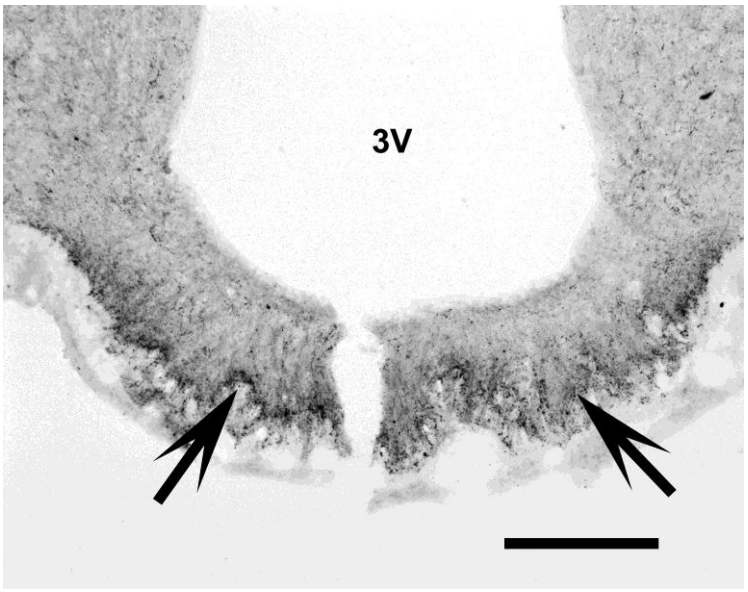

Fig. 30. Microphotograph shows ENK immunoreactivity in the ME of a lactating rat 8 hrs after removal of pups. Arrows show immunoreactive fibers. Abbreviation: 3V = third ventricle. Scale: 250μm.

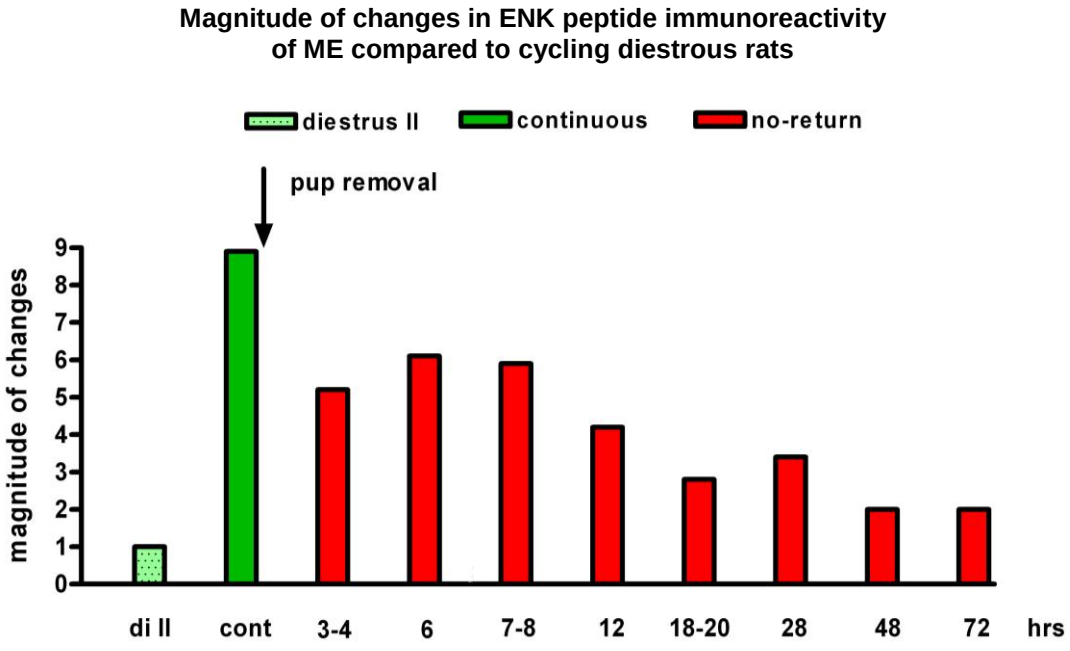

Fig. 31. Magnitude of changes in the ENK immunostaining in the various experimental groups compared to cycling diestrous rats. The 'no-return' bars show dams whose pups have been removed (and never returned). The green bar represents continuously lactating dams, and dotted green bar shows diestrous rats.

DOI:10.14753/SE.2012.1755

We also investigated the magnitude of changes in the of ENK and TH mRNA after pups were removed from lactating dams compared to one another (Fig. 32). The magnitude of change in TH mRNA expression is much greater than that of ENK. The removal of the suckling stimulus resulted in an 8 fold change from lactating levels by 3-4 hours and continued to rise throughout the experiment (24-28 hours after pup-removal), reaching a 25 fold increase. We do not have further time points for TH. ENK mRNA expression following the removal of pups only increased by 2.5 fold at 3-4 hours and peaked at 4 fold at 7-8 hours. Finally, 48 and 72 hours later, mRNA levels were already lower than in lactating dams. Diestrous levels are much below the lactating levels.

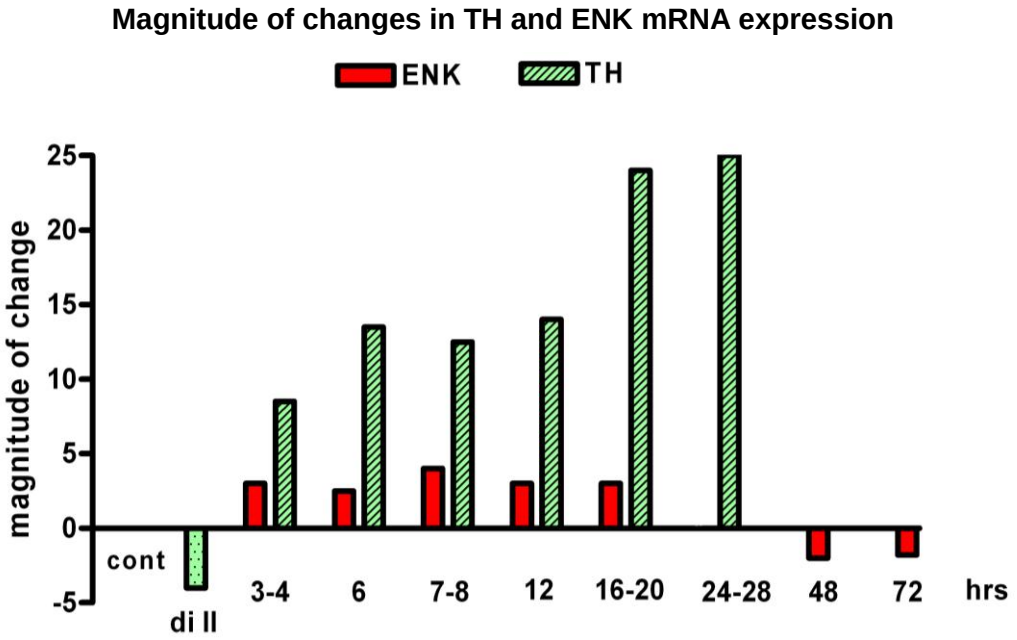

Fig. 32. The graph demonstrates the magnitude in the rise (or fall) of the overall expression of TH and ENK mRNA after pup removal compared to continuously lactating levels. Value of the continuously lactating group is considered the baseline (0), to better demonstrate the fall in mRNA expression (negative values).

DOI:10.14753/SE.2012.1755

5.3. Autonomic innervation of the mammary gland

5.3.1. Specificity test for retrograde transportation of virus

The virus was injected at right side into the first and second nipples and the underlying mammary glands. Independent of the length of survival time GFP labeling was observed in the dorsal root ganglia at the level of Th2-6 segments (Fig 33A) but not in the dorsal rootlets and dorsal horn of the spinal cord. It means that the applied virus was exclusively transported in a retrograde manner because the central axon of the pseudounipolar neurons could not transport the virus to the next member of the sensory neuronal chain.

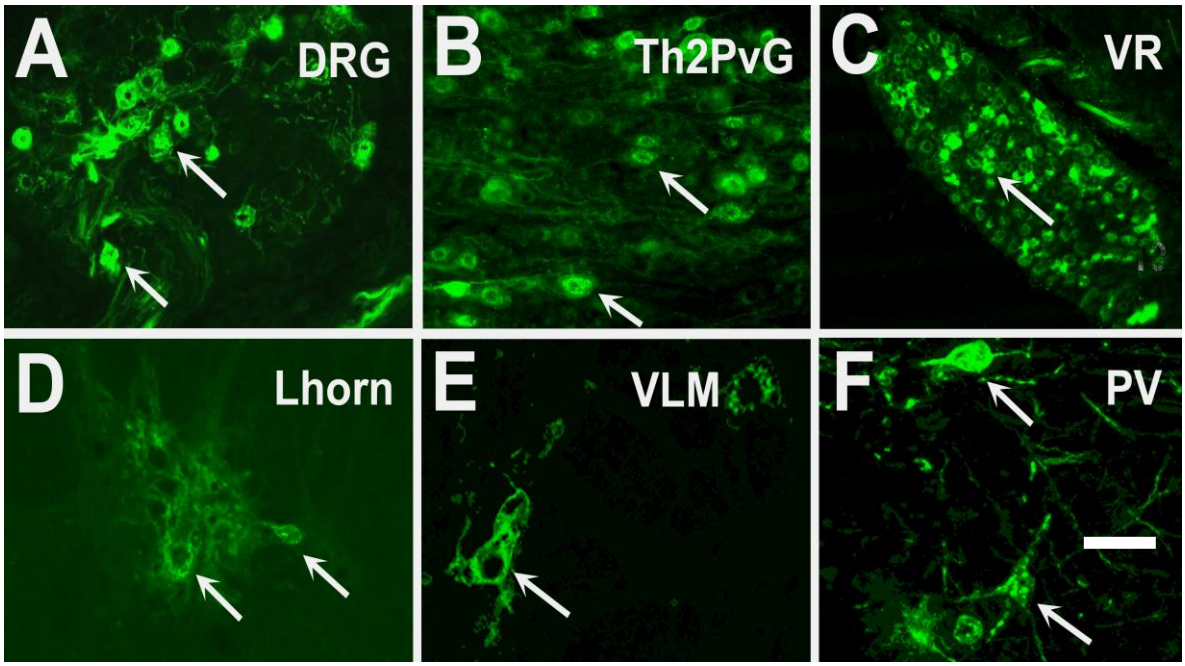

Fig. 33. Microphotographs demonstrating virus labeling at ipsilateral side of various level of the nervous system after right nipple and mammary gland inoculation (A-F) **A**. GFP labeling in the dorsal root ganglion (DRG). **B.** GFP labeling in the 2nd paravertebral ganglion (Th2PvG). **C.** GFP labeled fibers in a ventral rootlet. (VR). **D.** GFP labeling in the lateral horn (Lhorn) of the 2nd thoracic segment of the spinal cord. **E.** GFP neurons in the ventrolateral medulla (VLM). **F.** GFP labeling in the hypothalamic paraventricular nucleus (PV). Scale: 50μm in A, B and D, 25μm in E and F, and 10μm in C.

DOI:10.14753/SE.2012.1755

5.3.2. Virus labeling at the various levels of the nervous system

When the animals were sacrificed two days after the injection, GFP labeling was observed in the ipsilateral upper thoracic paravertebral sympathetic ganglia (PvG). Fig. 33B shows the microphotograph of Th2PvG. Ventral rootlets (VR) at the corresponding levels were also labeled (Fig. 33C). In the spinal cord labeling was observed in the ipsilateral lateral horn (Fig. 33D). A considerable number of labeled cells were seen in Th2-Th5 segments, and just a few in Th6 segment. Below this level there was no labeling in the lateral horn. Because the virus was transported in a retrograde manner, a few motor neurons were also labeled by GFP if some skeletal muscle fibers under the mammary gland were inoculated by the virus.

When the animals were sacrificed three days after the injection, a few labeled neuronal cell bodies appeared in the brain stem and the hypothalamus. When the animals were sacrificed four days later many labeled cells were seen at both sides in the VLM (Fig. 33E, ipsilateral side) and scattered cells in other brain stem regions including locus ceruleus, raphe nuclei, periaqueductal gray matter. Many labeled cells was observed in the PV at the ipsilateral side (Fig. 33F) and only a few at the contralateral side.

5.3.3. Chemical characterization of the virus labeled neuronal perikarya in PvG

In the PvG there were many small size DBH immunoreactive and a few large VAChT immunoreactive neurons and a dense network of VAChT immunoreactive fibers. A subpopulation of GFP conjugated virus labeled perikarya showed DBH (Fig.34A) or VAChT immunoreactivity (Fig. 34B). The DBH immunoreactive material filled out the cells; however, the VAChT immunoreactivity formed small granules mainly at the periphery of the cells, on the inner surface of the cell membrane.

DOI:10.14753/SE.2012.1755

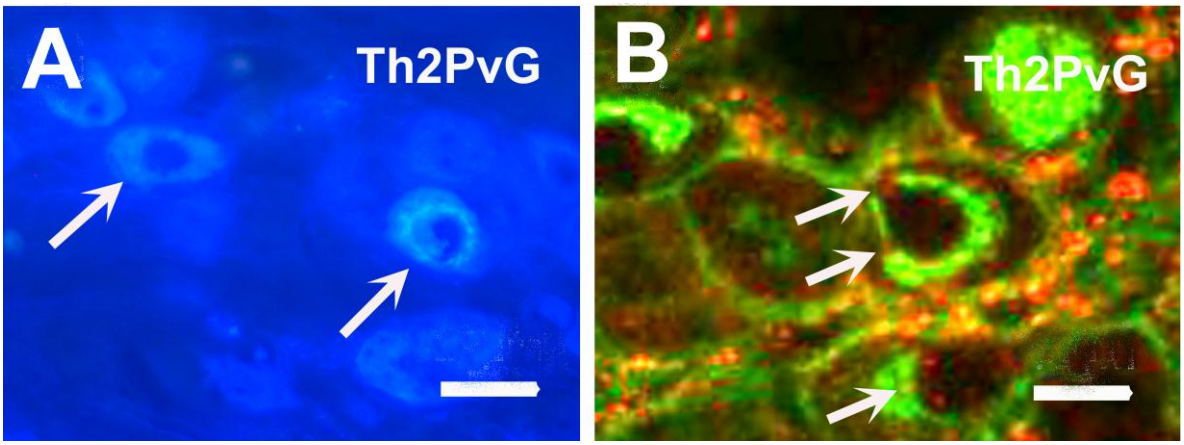

Fig. 34. Microphotographs demonstrating colocalization between virus labeling and DBH (**A**) and virus labeling and VAChT immunoreactivities at ipsilateral side after right nipple and mammary gland inoculation (**B**). Arrows indicate double labeled cells. Scale: 25μm.

5.3.4. Chemical characterization of neuronal cell bodies in the lateral horn and PV

In the lateral horn the GFP labeled neurons also showed VAChT immunoreactivity (Fig. 35). We did not observed DBH immunoreactive perikarya in this region, but a very dense DBH positive fiber network was present. In the VLM the virus labeled neurons were immunopositive for DBH (not shown). In the PV a subpopulation of GFP labeled neurons also showed OXY immunoreactivity (Fig. 36).

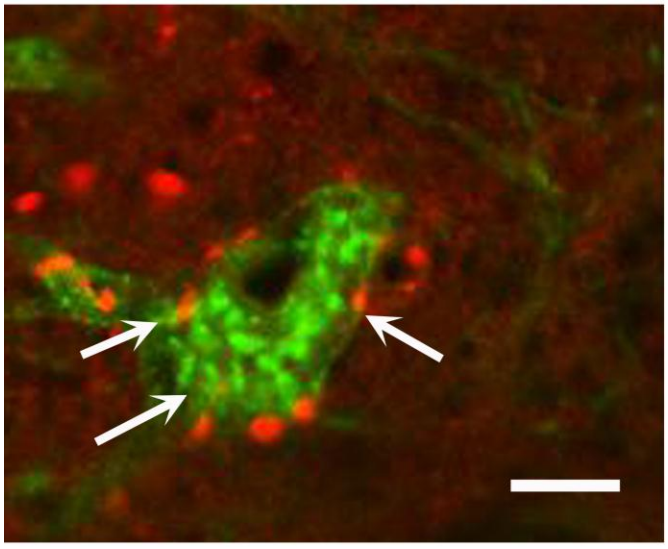

Fig. 35. Microphotographs demonstrating colocalization between virus labeling (green) and VAChT immunoreactivity (red, arrows) in the lateral horn at ipsilateral side after right nipples and mammary gland inoculation. Scale: 10μm.

DOI:10.14753/SE.2012.1755

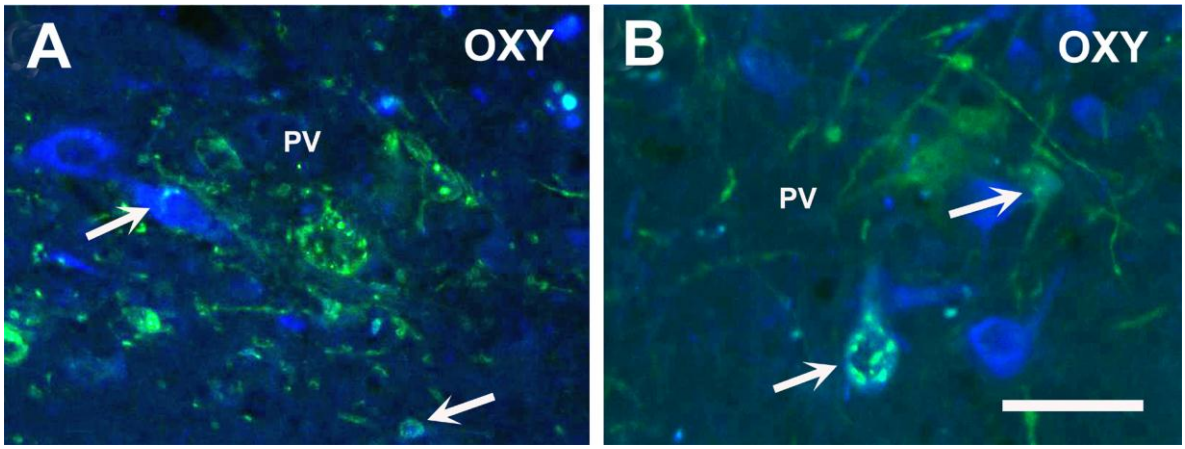

Fig. 36. Microphotographs demonstrating colocalization between virus labeling (green) and OXY immunoreactivity in the PV. **A** and **B.** Two details of the PV show OXY immunopositive cells (blue color) which also contain virus (green colour). Where the two colours overlap each other the colour is greenish-yellow. Abbreviation: PV = paraventricular nucleus; OXY = oxytocin. Scale: 75μm.

5.3.5. Chemical characterization of the nerve fibers in the mammary gland

S-100 immunostaining revealed all types of peripheral nerve fibers in the mammary gland and nipple. Some nerve fibers were present in the wall of vessels and in the connective tissue. We have also observed S-100 immunoreactivity surrounding the alveoli. The myoepithelial cells showed this relatively pale S-100 staining. Some of fibers were CGRP immunoreactive and were only observed in the connective tissue of the nipple and under its epithelium, but not in the mammary gland (Fig. 37).

DOI:10.14753/SE.2012.1755

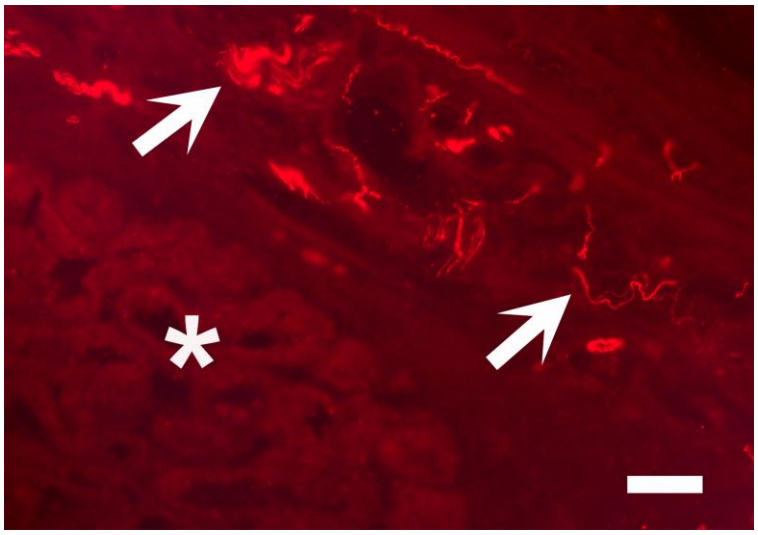

Fig. 37. Microphotographs demonstrating calcitonin gene-related peptide (CGRP) fibers in the nipple, but not in the underlying mammary gland. Arrows indicate immunoreactive fibers, * shows negative alveoli. Scale: 20μm.

Nerve fibers in the wall of vessels of the nipple and mammary gland showed DBH immunoreactivity (Fig. 38); however, DBH fibers were not present between the alveoli and in the wall of ducts.

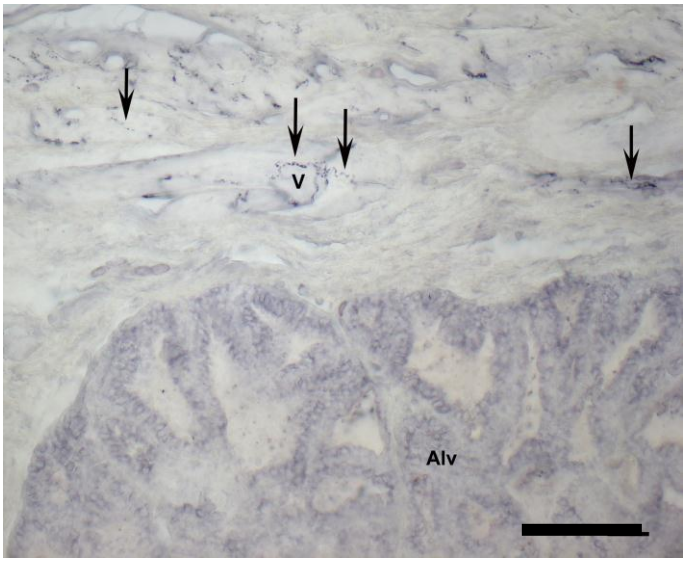

Fig. 38. Microphotographs demonstrating DBH immunoreactive fibers in the wall of vessels (v) of the mammary gland (indicated by arrows). The alveoli (Alv) did not innervated by DBH fibers. Scale: 50μm.

DOI:10.14753/SE.2012.1755

VAChT immunoreactive fibers were observed neither in the alveoli and ducts of the mammary gland nor in the wall of vessels (Fig. 39A and B); however, sweat glands in the neighbourhood of the nipple were innervated by VAChT immunoreactive fibers (not shown).

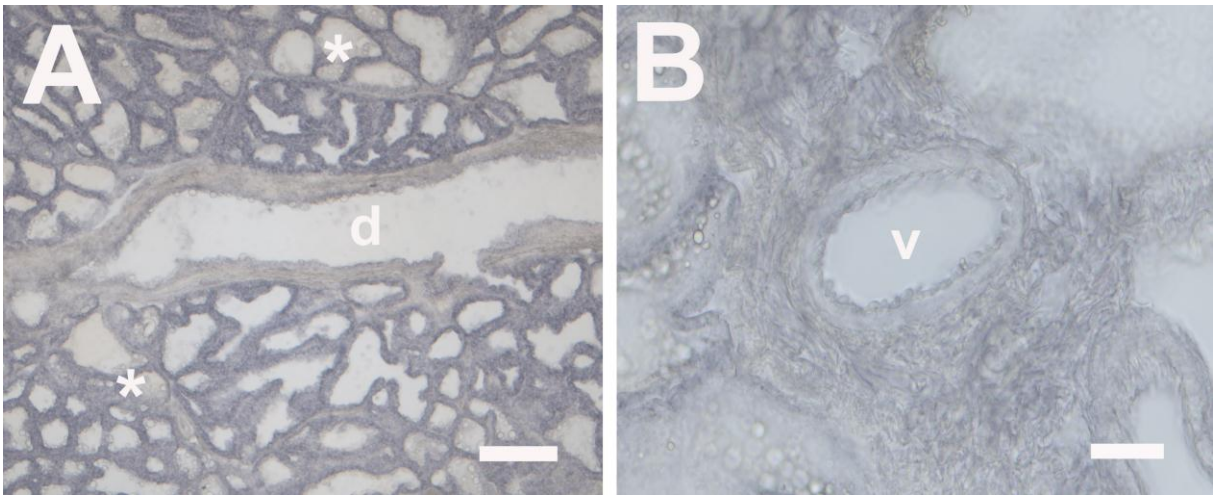

Fig. 39. Microphotographs demonstrating VAChT immunostaining in the mammary gland. Immunoreactive fibers were not observed either in the vessels or around the alveoli and the wall of ducts. Abbreviations: d = duct; v = vessel; * = asterisk indicate alveoli. Scale: 50μm and 20μm in B.

DOI:10.14753/SE.2012.1755

6. DISCUSSION

6.1. Morphological findings for suckling induced PRL release pathway

Our BDA injections confined to the PPN failed to label any fibers in the ARC, but did label cells just in the vicinity of ARC and in the VMN. Although the VMN has been suggested to play a role in the regulation of PRL in the turkey (Youngren et al 2002), it is unlikely that this nucleus relays suckling stimulus to TIDA neurons via the ARC in mammals.

The PPN, however, could still be a very important nucleus in the process of lactation, especially since it has been shown to be activated by the suckling stimulus as well as by exteroceptive stimuli from pups such as visual, olfactory and auditory in the absence of suckling (Li et al 1999a). Such stimuli become especially important in later stages of lactation in maintaining milk supply (Febo et al 2008). Unilateral chemical or radiofrequency lesioning of the PPN on pp 7 showed impairment to lactation; however, it did not affect PRL secretion and only slightly impaired maternal aggression, while other factors of maternal behavior remained unaffected. The author's conclusion was that the effect had to be attributed to deficient oxytocinergic activity (Factor et al 1993; Hansen and Kohler 1984). Another study showed that hemitransection of the midbrain tegmentum, including the region of PPN, only blocks the milk ejection reflex from contralateral suckling (Wang et al 1996a) and bilateral suckling still remains more effective than unilateral suckling in eliciting milk let-down after these lesions (Wang et al 1995; 1996a; 1996b). The results of our tracing experiments from the PPN suggest that there is a PPN-VMN projection. The VMN has been known to play a role in the control of eating, as well as certain aspects of behavior. Bilateral lesions of VMN in animals result in overeating (hyperphagia) and extreme obesity as well as a chronically irritable mood and increase in aggressive behavior, also referred to as hypothalamic rage (Factor et al 1993; Grundman et al 2005). This could mean that the PPN is involved in conveying the suckling stimulus to the VMN, and thus promotes hyperphagia, which is a typical metabolic response during nursing.

DOI:10.14753/SE.2012.1755

The SPFpc is a subnucleus at the border of the midbrain and the posterior intralaminar thalamus. It consists of horizontally oriented cells and extends rostromedial to caudolateral direction and overlies the medial lemniscus (Ledoux et al 1987; Coolen et al 2003; Veening et al 1998). BDA injections confined to the SPFpc did label fibers in the ARC. The fibers were mostly found in the ventrolateral part of the ARC, with very few seen in the dorsomedial part. Double labeling with TH revealed that the cells contacted by these fibers are not TIDA cells. Therefore, these neurons of the ARC are probably just a relay population to TIDA cells. Previous experiments suggest that about 70% percent of TIDA neurons are innervated by DYN containing axons (Fitzsimmons et al 1992). We hypothesized that the BDA labeled axons in the ventrolateral part of the ARC, originating in the SPFpc, are actually terminating on DYN neurons. However, BDA-DYN double labeled immunocytochemistry did prove this right. Inspite of this fact it is not excluded that the exact circuit within the ARC thus still needs to be explored.

Our retrograde tracing experiments suggest that there is a direct connection between ARC and SPFpc. The injection of the retrograde tracer FG confined to the ARC resulted in labeled cells in the SPFpc of the midbrain, ventral and medial to the PPN. Fos studies show this nucleus to be associated with mating behavior, specifically with ejaculation in male rats and vaginocervical stimulation in females (Coolen et al 1996). This research group phenotypically characterized the SPFpc and found that the nucleus has a medial subdivision containing dense GAL-immunoreactive fibers, a lateral subdivision which contains CGRP immunoreactive fibers and neurons, and an intermediate subdivision, which only contains a few labeled fibers or neurons for either GAL or CGRP. Based on these stainings; however, the lateral portion of the SPFpc seems to blend into the PPN (Coolen et al 2003).

TIP39 is a recently characterized ligand of the parathyroid hormone 2 receptor. Dobolyi and his coworkers (2003) have mapped the expression of this peptide in the rat brain and found that a major population of TIP39 neurons resides in the SPFpc. Double immunostaining also showed that many TIP39 cells in the SPFpc are CGRP positive as well. Our retrograde FG injections do label TIP39 as well as CGRP positive neurons in the SPFpc, just over the medial lemniscus.

DOI:10.14753/SE.2012.1755

Our findings well correlates with the previous observation of Bodnár and her collaborators (2002). They have demonstrated that frontal deafferentations located in the anterior and posterior hypothalamus prevent the suckling induced PRL release. These interventions interrupted the connections between the midbrain and the hypothalamic PV abolishing their serotoninergic input from the brain stem. In control animals PV receives a rich serotoninergic innervation. After deafferentations immunocytochemistry revealed that there were almost no serotoninergic fibers and terminals in the PV. These lactating animals did not show a rise in plasma PRL levels upon suckling stimulation. The same research group emphasized the role of glutamaterg innervation of the raphe nucleus in SIPR (Bodnár et al 2009). Microinjection of non-NMDA receptor antagonist into the dorsal raphe nucleus significantly attenuated the PRL release upon suckling stimulus.

In summary, the previously proposed midbrain nucleus that plays a role in the regulation of PRL secretion via the ARC during lactation does not seem to be the PPN. Instead, we propose that the adjacent SPFpc may be the relay of the suckling stimulus to the ARC in lactating rats. We used non-lactating rats in our morphological studies. It is not excluded that in lactating rats fibers from the mesencephalon can reach the TIDA neurons directly. However, in the ARC, we still do not know the exact neuronal circuit involved in conveying the stimulus to the TIDA population. Our study suggests that additional relay neurons reside in the ventrolateral ARC. Fig. 40 shows our finding about the pathway of SIPR. On the basis of previous (Li et al 1999b) and our recent studies, we propose that the pathway of SIPR consists of 6 neurons: dorsal root ganglion, posterior horn of the spinal cord (Rexed lamina 4-5), lateral cervical nucleus, SPFpc, ventrolateral ARC, and finally the TIDA neurons. One more relay neuron in ARC is also supposed.

DOI:10.14753/SE.2012.1755

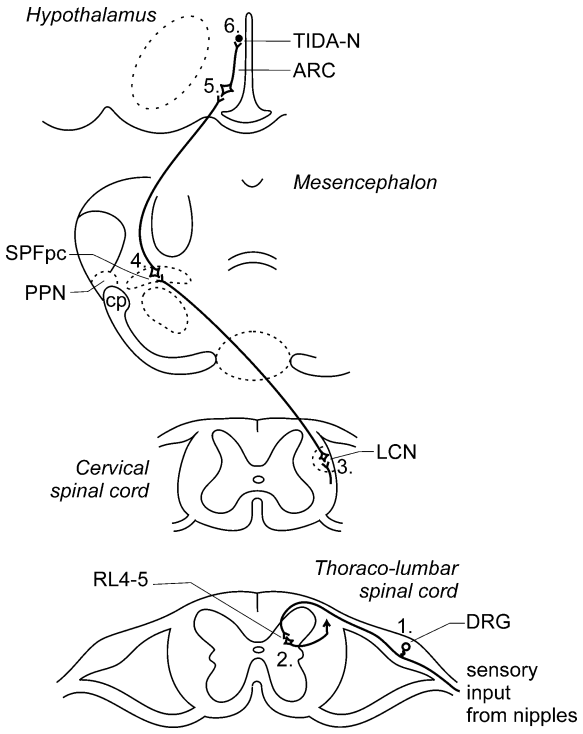

Fig. 40. Our proposal of the pathway of suckling induced PRL release. The pathway is composed of 6 neurons from the primary sensory to TIDA neurons. Abbreviations: ARC = arcuate nucleus; cp = cerebral peduncle; DRG = dorsal root ganglion; LCN = lateral cervical nucleus; PPN = peripeduncular nucleus; RL4-5 = Rexed laminae 4-5; SPFpc = subparafascicular parvocellular nucleus; TIDA-N = tuberoinfundibular dopaminergic neurons.

6.2. Physiological findings: Effect of suckling stimulus on TH and ENK

The physiological results confirm the hypothesis that the suckling stimulus is an important regulator of TH expression in TIDA neurons. In cyclic diestrous rats the TH mRNA level is high, about ten times higher than in continuously lactating rats (Wang et al, 1993). A previous study (Berghorn et al 1995) showed that the increase of nuclear TH mRNA was evident as early as 1.5 hours after the removal of pups and that the heteronuclear RNA levels peaked at 3 hours, and then declined as cytoplasmic mRNA increased. Thus the selection of a 4 hour-removal period prior to pup return in the present study represented a time after which the TIDA neurons clearly were in the process of up-regulating TH.

DOI:10.14753/SE.2012.1755

The results suggest that this up-regulation of TH mRNA can not be disrupted immediately if pups are returned and the neuronal input from the nipples to the ARC is reestablished. From our data it seems that the program of transcriptional up-regulation begins to very slightly subside in 10-12 hours after pups are returned and it is significantly lower in 16 hours than in the group where the pups were not returned. TH mRNA levels, however, stayed high for the duration of the experiment regardless of the resumption of suckling. Although the mRNA levels, found in dams that had not received their pups back, rose to higher levels than in dams that received their pups back and was significantly higher than in continuosly suckling controls. In theory we would have expected the decline in TH mRNA in pup-returned dams to the levels of continuously suckling dams with time based on the 6 hr half-life described for TH mRNA (Maurer and Wray 1997). Our observation indicates that re-suckling alters the stability of the TH mRNA producing machinary after being awakened by pup removal. One factor that could distinguish natural patterns of suckling from those after pups were removed could be the adrenocorticotrop hormone (ACTH)-corticosteroid axis. It was previously described that the suckling stimulus induces ACTH response. In a recent publication (Oláh et al 2009) it was found that in lactating dams the concentration of ACTH was higher in the intermediate than in the anterior lobe, and the inhibition of DA biosynthesis by α-methyl-parathyrosine or blockade of D2 receptors by domperidone enhanced the plasma ACTH level in an hour but did not influence the α-melanocyte stimulating hormone levels. In non-lactating (ovariectomized and ovariectomized+estradiol replaced) rats the above-mentioned drugs enhanced the α-MSH, but did not influence the ACTH levels. When the pups are removed then returned both PRL and ACTH plasma levels enhanced and bromocriptin prevented the elevation of both hormones. However, it is known that during lactation a number of stressors are less effective than in non-lactating conditions (Kehoe et al 1992).

To get a better understanding of how the number of cells that express TH mRNA changes with the OD of mRNA, we have to view the results together. Though the number of cells containing TH mRNA clusters did increase in number, this rise became significant only 24 hours after the termination of the suckling stimulus (pup-removal). However, by measuring the OD of expressed mRNA, we found that it has already significantly increased

DOI:10.14753/SE.2012.1755

6 hours after the termination of suckling. It means that the enhancement of the number of cells expressing TH mRNA and the intensity of the expression are not completely parallel processes.

Endogenous opioids have also been implicated in the regulation of suckling-induced PRL secretion during lactation (Arbogast and Voogt 1998). A possible candidate could be ENK, a δ receptor agonist. The ARC nucleus contains scattered ENK immunoreactive neurons in cycling animals, but during lactation, ENK expression is strongly enhanced in TIDA neurons (Ciofi et al 1993; Merchenthaler 1993). Our results examining ENK expression in the TIDA neurons also show that during continuous suckling, levels of ENK mRNA are significantly higher than in cycling females. The pup-removal produced a further increase in OD, similarly to the cell counts, then both declined. The levels of ENK peptide in the ME started to drop earlier than the mRNA in the ARC, already four hours after the pup removal; however, the ENK mRNA only in 8 hours after pup removal. None of these parameters reached the control diestrous levels.

What kind of mechanism is responsible for maintaining ENK synthesis in TIDA neurons so long after the cessation of suckling is unclear. The up-regulation of ENK is thought to be the result of the hyperprolactinemia of lactation (Merchenthaler, 1994; Merchenthaler et al 1995). A variety of experimental paradigms show that elevated serum PRL levels are accompanied by up-regulation of ENK. Certainly the levels of PRL fall rapidly (about 2 hours) after suckling ceases and the normal peaks in PRL secretion that accompany estrous cyclicity are not sufficient to prompt significant co-expression of ENK in TIDA neurons (Grosvenor et al 1979).

On the basis of the results it was concluded that the up-regulation of DA synthesis after termination of suckling is an active process rather than a simple switch prompted by brief interruption in suckling (Fig. 41A). Our results support Hypothesis I. This regulatory mechanism is efficient at pp 10 in rats. For ENK, we can say that lactation does result in some increase in its mRNA expression; however, the translation of the message into peptide is what is truly striking. During continuous lactation ENK protein levels are high in the ME and it is released into the portal circulation in larger amounts a few hours after suckling stops (Fig. 41A). Do TIDA neurons respond to this by increasing ENK mRNA

DOI:10.14753/SE.2012.1755

expression to allow additional ENK to be produced, or is this just a response to the stress of pup-removal? If the goal is to produce more ENK, perhaps ENK really does have a protective role against the inhibitory effect of TH (and consequently DA) whose mRNA levels also increase following the removal of the suckling stimulus. The return of pups interrupts the up-regulation of TH mRNA, but mRNA levels remain high even a day after the resumption of lactation. ENK mRNA also remains high during this time (Fig. 41B).

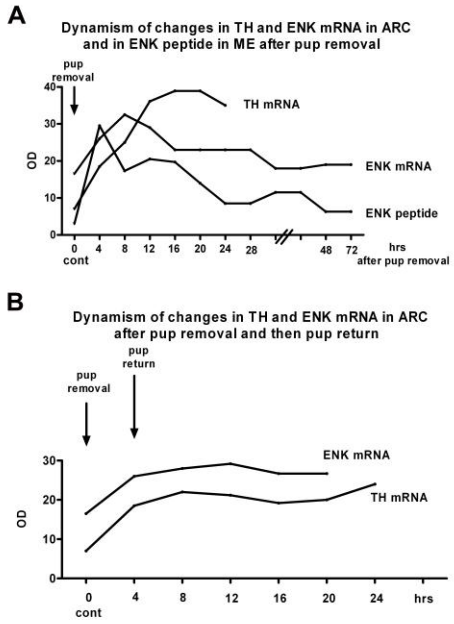

Fig. 41. Dynamism of the changes in the optical density of TH and ENK mRNA in the ARC and optical density of the ENK peptide immunostaining in the ME after pup removal, and then pup-return after four hour separation.

In summary, we may say that, on one hand, after pup-removal both TH and ENK mRNA are upregulated, 8 hours later TH mRNA keeps to increase, but ENK mRNA shows opposite changes as TH mRNA, it starts to decrease. These changes show that the temporal program of TH and ENK regulation is different in the case of pup removal, and ENK response is more slow than that of TH. Upon pup return four hours later both TH and ENK mRNA remain higher than in continuously lactating rats, the curves in Fig. 41B showing the OD of TH and ENK mRNA run parallel that is the regulation of both TH and ENK shows similar temporal program at least for 24 hours.

DOI:10.14753/SE.2012.1755

6.3. Morphological findings for the autonomic innervation of the mammary gland

Our virus labeling shows that the autonomic innervation of the rat mammary gland follows the general rules. Postganglionic neuronal cell bodies are present in the PvG. The preganglionic neuronal cell bodies are present in the corresponding part of the lateral horn of the spinal cord. The neurons in the lateral horn receive afferents from the brain stem and hypothalamic PV. Injection of the GFP labeled virus, spreading exclusively in a retrograde manner, in the 1st and 2nd nipples and underlying mammary gland resulted in labeling at ipsilateral side in the first order neurons that is in the paravertebral sympathetic trunk then in the second order neurons in the VRs and in the lateral horn at Th1-6 segments. Three days after inoculation the labeling appeared in the brain stem and in the hypothalamic PV. Above the spinal cord the labeling was bilateral. However, the majority of the labeled cells were present at the ipsilateral side. These findings well correlate with those demonstrated by Gerendai and her coworkers (2001). However, their virus was transported in both ante- and retrograde manners. There is some discrepancy in the number of labeled cells. It can be explained by the fact that we used much thinner slides (20μm thick) than the above mentioned authors, and we injected only two nipples, not three and the amount of injected saline containing virus was smaller (2μl v 5μl of $8x10^8$ PFU).

Chemical characterization of the virus labeled neurons in the PV revealed that the subpopulation of these neurons synthetize OXY. It is well known from the literature that OXY release is modulated by various stressors, such as immobilization and psychological stress (Carter and Lightman 1987; Sanders et al 1990). It is also demonstrated that acute physical and mental stresses can impare the milk ejection reflex by reducing the release of OXY in breastfeeding women (Dewey 2001). The cited authors in the 'Introduction' suggest that the inhibition of milk yield at the beginning of suckling in rats is mediated by a reflex pathway closed in the central nervous system and the last member of this neuronal chain is probably catecholaminergic. It was published by Michaloudi and his coworkers (1997) that the oxytocinergic magnocellular neuronal somata were heavily innervated by noradrenergic fibers. The analysis was carried out on semithin sections of rat hypothalamus. The density of noradrenergic varicosities was similar in male and intact and

DOI:10.14753/SE.2012.1755

lactating female rats. Similar results were obtained by Semeniken and his coworkers (2009) who used human tissues. Double labeling immunohistochemistry revealed intimate association between TH immunoreactive fibers and OXY immunoreactive neurons. This juxtaposition suggests synapses between the two elements. Our virus labeling and double labeling immunohistochemistry clearly show that the first-order neurons of the descending pathway from the hypothalamus to the mammary gland is partially oxytocinergic and it is also clear from the literature that these neurons are heavily innervated by noradrenergic fibers (Semeniken et al 2009). This finding well correlates with the results that central administration of β-adrenergic blocker propranolol enhanced the milk yield through relieving the OXY provoked ductal constriction (Morales et al 2001). It means that in this condition noradrenalin is inhibitory for the OXY release.

The further characterization of the descending neuronal chain to the mammary gland and the nipple confirmed that the second order neurons in the lateral horn of the spinal cord are cholinergic. The third order neurons in the PvG labeled by virus are also noradrenergic .

In the second part of the experiment we studied the innervation of the nipple and mammary gland. S-100 is a astrocyte-derived protein. Originally it was isolated from bovine central nervous system; however, it is also present in the peripheral nervous system. It was demonstrated in the satellite cells of sensory, sympathetic and enteric ganglia, supporting cells of adrenal medulla, Schwann cells of nerve trunks and Schwann-related cells of sensory corpuscules (Gonzales-Martinez et al 2003). Immunohistochemistry revealed that S-100 immunoreactivity is also present in the myoepithelial cells (Grandi et al 200). We could confirm these observations in the mammary gland and we have further examined what is the chemical character of S-100 fibers in this gland. We have also used CGRP immunostaining because among other peptides (substance-P, and neuropeptide K, but not vasoactive intestinal polypeptide, peptide histidine isoleucine and neuropeptide Y) CGRP was demonstrated in the sensory nerve endings of the nipple by Pinho and Gulbenkian (2007). We did not find CGRP fibers between the alveoli or the wall of ducts, just in the connective tissue of the nipple, in the wall of vessels and under the epithelium.

DOI:10.14753/SE.2012.1755

We also tried to identify autonomic fibers in the nipple, between the alveoli and in the wall of ducts. DBH immunoreactive fibers were only present in the wall of the vessels. It explain the colocalization between the virus labeling and DBH immunoreactive neurons in the PvG and also explains the early observation of Grosvenor and Findlay (1968) that the denervation influence the fluid flow into the mammary gland. We did not find DBH or VAChT immunoreactive fibers in the mammary gland, either between the alveoli or in the wall of the ducts. The observed colocalization between the virus labeling and VAChT immunoreactivity in the PvG explained by the inoculation of sweat glands in the neighbourhood of nipples.

In summary, it was concluded that the descending pathway, which provide the autonomic innervation of the structures of nipple and the mammary gland and may be involved in the regulation of milk yield in rats, is composed of at least three neurons (Fig. 42). The first-order neurons are mainly located in hypothalamic PV, and these neurons are partially oxytocinergic and they receive noradrenergic input. First-order neurons may also occur in some brain stem nuclei. These cell groups are known to be noradrenergic. The second order neurons are present in the lateral horn, and these neurons are cholinergic. The last (third-order) neurons are located in the paravertebral sympathetic trunk and these neurons are noradrenergic. Noradrenergic fibers innervate vessels and in this way may influence the blood supply of the mammary gland. Neither noradrenergic nor cholinergic fibers were seen in the wall of ducts and between the alveoli.

There are two new findings in the third part of this work:

1) the virus labeled neurons in PV which axons descend to the lateral horn of the spinal cord may also synthetize OXY.

2) between the alveoli and ducts of the mammary gland we were not able to detect noradrenergic (showing DBH immunoreactivity) or cholinergic (showing VAChT immunoreactivity) fibers in rat, DBH fibers were only present in the wall of vessels.

DOI:10.14753/SE.2012.1755

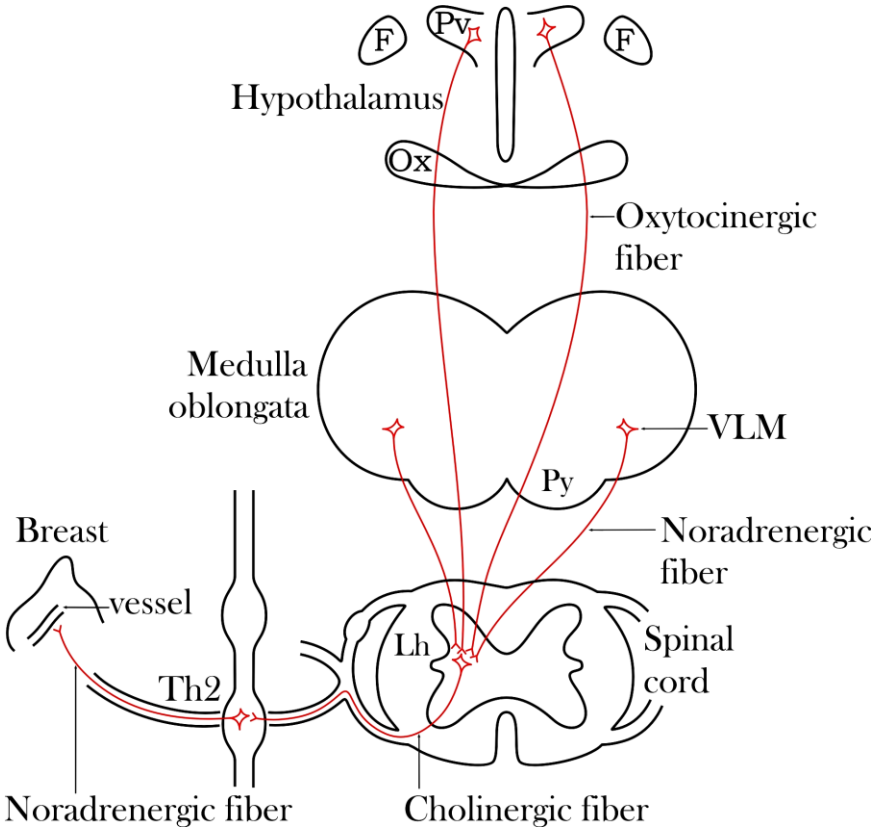

Fig. 42. Schematic illustration of the descending neuronal pathway from the PV and VLM to the mammary region and the neurotransmitters and peptides used by the neurons. Abbreviations: F = fornix; Lh = lateral horn; OX = optic chiasm; Pv = paraventricular nucleus; Py = pyramid, Th2 = 2nd thoracic paravertebral ganglion; VLM = ventrolateral medulla.

Our results provide morphological basis of the previous theory that the milk yield at the beginning of suckling is mainly influenced by central effect of noradrenergic input and at this level influence the OXY release from the posterior pituitary to the general circulation. An alternative theory is also arised: descending oxytocinergic influence may modify the function of the lateral horn neurons. It was supposed that the balance of supranuclear oxytocinergic input from the PV neurons through the descending noradrenergic input, which regulate the blood supply, and hormonal oxytocinergic input from the posterior pituitary may set in the milk yield of the rat mammary gland at the beginning of suckling.

DOI:10.14753/SE.2012.1755

7. BRIEF CONCLUSION OF THE THREE EXPERIMENTS

I. An ascending pathway from the nipple to the hypothalamic TIDA neurons regulates the PRL secretion which induces milk secretion. In the present work the neuronal chain of this pathway was further clarified. SPFpc was identified as one of the relay station.

II. A descending autonomic pathway regulates the milk yield at the beginning of suckling. This pathway is under noradrenergic influence. In this work the neurotransmitters of this pathway was further clarified. In the mammary gland in rats only the vessels are innervated by autonomic fibers which use noradrenaline as neurotransmitter.

III. TH and ENK mRNA levels in the ARC of lactating rats run parallel after removal of pups from the mother that is the regulation of both TH and ENK shows similar temporal program at least for 24 hours after removal of pups.

DOI:10.14753/SE.2012.1755

8. NEW RESULTS

1. The ascending sensory pathway from the mammary gland is composed of at least six neurons. Previously it was proposed that the mesencephalic relay neurons are located in the PPN. On the basis of our results it was suggested that the mesencephalic relay is not only the PPN, but the SPFpc as well.

2. It was also demonstrated at the first time that the neurons of SPFpc project to the ventrolateral part of ARC and this ascending neurons are TIP immunoreactive.

3. In the ARC we have found DYN immunoreactive neurons which showed interaction with ascending fibers from the SPFpc. This result suggests that DYN neurons relay the suckling stimuli to TIDA neurons.

4. Our present results show that the up-regulation of DA synthesis after suckling termination is an active process rather than a simple switch prompted by brief interruption in suckling.

5. Lactation does result in some increase in ENK mRNA expression (both in the number of labeled cells and in the density of labeling). During continuous lactation ENK protein levels are high in the ME and it is released into the portal circulation in large amount a few hours after suckling stops. The role of this high ENK release may be protective response to pup removal.

6. The descending autonomic pathway which innervates the blood vessels of the mammary gland and influences the milk yield at the beginning of suckling is composed of three neurons. The first order neurons use OXY or noradrenaline, the second order neurons use acethylcholine and the third order neurons use noradrenaline neurotransmitter.

DOI:10.14753/SE.2012.1755

9. SUMMARY

The neuronal pathways, through which prolactin secretion is regulated during lactation, have still not been fully explored. Studies indicate that the suckling stimulus from the nipple travels through the spinal cord, the brain stem, and then reaches the hypothalamus. One of the focus of the present studies was to further explore the neuronal connections between the brain stem and the arcuate nucleus that might be involved in suckling-induced prolactin release. Ante- and retrograde tracing techniques were used. To chemically characterize the explored neurons, neuropeptide immunohistochemistry was applied. Previous studies have indicated that the peripeduncular nucleus is a relay of the suckling stimulus in the midbrain, conveying the information to the hypothalamus. In our experiments we have found an additional cell group in the subparafascicular parvocellular nucleus located just behind the posterior thalamus that projects to the arcuate neurons. The injection of the retrograde tracer into the ventrolateral part of the arcuate nucleus labeled cells in the lateral subdivision of the subparafascicular parvocellular nucleus. Anterograde tracing from the subparafascicular parvocellular nucleus resulted in fiber labeling in the arcuate nucleus in close apposition with dynorphin immunopositive neurons. It is also known that the dynorphine neurons innervate the tuberoinfundibular dopaminergic (TIDA) neurons. Double labeling revealed that a subpopulations of the subparafascicular parvocellular neurons projecting to the arcuate nucleus contained tuberoinfundibular peptide of 39 residues or calcitonin gene-related peptide. The presented findings suggest that the ascending fibers from the subparafascicular parvocellular nucleus might be on the pathway involved in the suckling-induced prolactin release.

Dopamine (DA) and enkephalin (ENK) release from TIDA neurons into the hypophysial portal circulation is basically different under non-lactating and lactating conditions. During lactation DA down- and ENK are up-regulated. The other aim of the present studies was to compare the effect of a brief interruption then resume of suckling on the temporal program of tyrosin hydroxylase (TH) (rate-limiting enzyme of dopamine synthesis) and ENK regulations in dams. On post partum day 10 we removed pups for 4 hrs and half of the dams had their pups returned for periods between 4 and 24 hrs to examine

DOI:10.14753/SE.2012.1755

whether brief interruption of suckling provokes full up-regulation of TH and down-regulation of ENK, and whether re-initiation of suckling limits the extent to which TH up- and ENK down-regulate. At the end of experiment the animals were decapitated. *In situ* hybridization was used to examine the expression of TH and ENK mRNA in arcuate nucleus and immunohistochemistry to demonstrate the ENK peptide in the median eminence. The results showed that, on one hand, the pup-removal induced TH up-regulation was not immediatelly interrupted by the reinitiation of suckling, it remained relatively high during the examination period (24 hrs). On the other hand, ENK expression also increased upon pup removal for 8 hrs and then started to slowly decline. Although, the amount of ENK peptid in the median eminence declined more rapidly. In the case of reinitiation of suckling both TH and ENK mRNA were up-regulated at least for a day. On the basis of our results it was concluded that after termination of suckling both TH and ENK were up-regulated, the decline of ENK expression delayed about 8 hrs, ENK expression responded more slowly than that of TH, and after reinitiation of suckling the temporal program of regulation of both TH and ENK expressions ran parallel in the first 24 hours.

In the third part of this work the autonomic innervation of the nipple and the mammary gland was investigated by transynaptic virus labeling and the members of the neuronal chain were chemically characterized. The virus labeling revealed that the first neurons are present in the hypothalamic paraventricular nucleus (PV) and the brain stem, mainly in the ventrolateral medulla at both sides, the second order neurons are present in the ipsilateral lateral horn of the spinal cord, and the third order neurons are present in the paravertebral synpathetic ganglia. A subpopulation of the virus labeled neurons in PV also showed oxytocin immunoreactivity, the neurons in the ventrolateral medulla are noradrenergic, in the lateral horn the labeled neurons are cholinergic, in the sympathetic trunk they are adrenergic. Adrenergic neurons innervate vessels. The ducts and alveoli do not receive either adrenergic or cholinergic innervation in rats. For the low milk yield at the beginning of suckling not the peripheral innervation, but some events in the central nervous system are responsible which may influence the blood supply of the mammary gland. Oxytocinergic input from PV to the lateral horn may also be involves in this mechanism.

DOI:10.14753/SE.2012.1755

10. ÖSSZEFOGLALÁS

Az az idegpálya, amelyen keresztül a szopási stimulus prolaktin kidobást vált ki, még ma sem teljesen tisztázott. Morfológiai tanulmányaink célja volt további ismereteket szerezni erről a felszálló pályáról. Ante- és retrográd nyomkövető technikák segítségével Sprague-Dawley nőstény patkányokban vizsgáltuk, vajon a mesencephalonban korábban leírt peripeduncularis nucleus, amelyet a pálya egyik relé-állomásának tekintenek, és a n. arcuatus dopamin termelő sejtjei, a TIDA neuronok között van-e közvetlen neuronalis kapcsolat. Immunhisztokémiai módszerrel vizsgáltuk, hogy azok a neuronok, amelyek a nucleus arcuatusba küldik axonjaikat, milyen neuropeptideket tartalmaznak. Vizsgálataink arra utaltak, hogy a thalamus és a középagy határán elhelyezkedő subparafascicularis parvocelluláris mag tuberoinfundibilaris 39 peptid és a calcitonin gen related peptid immunreaktív neuronjai küldenek axon-végződéseket a nucleus arcuatus dinorfin tartalmú neuronjaihoz. Irodalmi adatokból ismert, hogy a dinorfin neuronok beidegzik a TIDA neuronokat, amelyeknek alapvető szerepe van a prolaktin szekréció szabályozásában.

A TIDA neuronok hypophysis portalis vérbe történő dopamin (DA) és enkefalin (ENK) ürítése alapvetően különbözik laktáló és nem laktáló nőstény patkányokban. Mind a DA-nak, mind az ENK-nak meghatározó szerepe van a prolaktin szekréció szabályozásában. Élettani kísérleteink célja volt összehasonlítani laktáló állatokban a szopás rövid idejű (4 óra) felfüggesztésének, majd újra indításának hatását a TIDA neuronok thyrosin hydroxyláz (TH) (a dopamin szintézis mértékét szabályozó enzim) és az ENK expressziójának időbeli lefolyására. A szülést követő 10. napon (post partum, pp) a kölyköket elvettük az anyáktól, majd az állatok fele 4 óra múlva visszakapta a kölyköket. Vizsgáltuk, hogy a szopás ilyen rövid idejű megszakítása provokálja-e a TH teljes up-regulációját és az ENK teljed down-regulációját, és vajon a szopás újraindítása leállítja-e a TH up- illetve ENK down-regulációt. A kísérlet végén az állatokat dekapitációval áldoztuk fel. *In situ* hybridizációs technikával vizsgáltuk a TH és az ENK mRNA expresszióját a n. arcuatus (A12 area) TIDA neuronjaiban, és immunhisztokémiai módszerrel vizsgáltuk az ENK peptidet az eminentia medianaban. Eredményeink arra utalnak, hogy a kölykök kimozdítását követő TH up-reguláció nem szakad meg azonnal a kölykök visszaadását

DOI:10.14753/SE.2012.1755

követően, viszonylag magas marad a 24 órás követési periódus alatt. Az ENK mRNA a várttal szemben még 8 óráig magas a kölykök kimozdítása után, majd lassan csökken. Ugyanakkor az eminentia mediana ENK peptid tartalma hamarabb csökken. A szopás újrakezdésekor mind a TH, mind az ENK mRNA magas legalább 24 órán át. Az eredményekből az a következtetés vonható le, hogy a szopás megszakítása után mind a TH mind az ENK up-regulált, az ENK expresszóo csökkenése 8 órát késik, lassabban válaszol a szopás megszakítására, mint a TH. A szopás újra indításakor mind a TH, mind az ENK expresszió up-regulációjának időbeli programja az első 24 órában parallel fut.

A munka harmadik része az emlőbimbó és az emlőmirigy autonom beidegzését vizsgálja transzszinaptikus virus jelöléssel. Immunhisztokémiai módszerrel jellemeztük a lánc egyes tagjait. A virus jelölés felfedezte, hogy a lánc első neuronjai a hypothalamikus n. paraventricularisban és a ventrolateralis medullában vannak mindkét oldalon., a másodrendű leszálló neuronok a gerincvelő oldalszarvában vannak, a harmadrendű neuronok a szimpatikus paravertebralis dúclácban. A virus jelölt neuronok egy része a n. paraventricularisban (PV) oxytocin immunreactivitást is mutat, a jelölt neuronok az oldalszarvban cholinergek, a sympathicus paravertebralis dúcokban főleg noradrenergek. Az noradrenerg neuronok ereket innerválnak. A kivezetőcsövek és alveolusok sem cholinerg, sem noradrenerg beidegzést nem kapnak laktáló patkányban. A szopás elején jelentkező alacsony tejhozamért nem a perifériás beidegzés, hanem központi idegrendszeri hatások felelősek, amelyek jelentősen befolyásolják az emlő vérellátását. A PV-ból az oldalszarvhoz leszálló oxytocinerg befolyás is szerepet játszhat ebben a mechanizmusban.

DOI:10.14753/SE.2012.1755

11. REFERENCES

Alam, S.K., Konno, T., Sahgal, N., Lu, L., Soares, M.J. Decidual cells produce a heparine-binding protein family cytokine with putative intrauterine regulatory actions. J. Biol. Chem. 283: 18957-18968, 2008.

Alam, S.M., Ain, R., Konno, T., Ho-Chen, J.K., Soares, M.J. The rat prolactin gene family locus: species-specific gene family expansion. Mamm. Genome 17: 858-877, 2006.

Andries, M., Tilemans, D., Denef, C. Isolation of cleaved prolactin variants that stimulate DNA synthesis in specific cell types in rat pituitary cell aggregates in culture. Biochem. J. 281: 393-400, 1992.

Anthony, P.K., Stoltz, R.A., Pucci, M.L., Powers, C.A. The 22 K variants of rat prolactin: evidence for identity to prolactin-(1-173), storage in secretory granules, and regulated release. Endocrinology 132: 806-814, 1993.

Arbogast, L.A., Voogt, J.L. Endogenous opioid peptides contribute to suckling-induced prolactin release by suppressing tyrosine hydroxylase activity and messenger ribonucleic acid levels in tuberoinfundibular dopaminergic neurons. Endocrinology 139: 2857-62, 1998.

Ben-Johnatan, N., Hnasko, R. Dopamine as a prolactin (PRL) inhibitor. Endocr. Rev. 22: 724-63, 2001.

Ben-Jonathan, N., Neill, M.A., Arbogast, L.A., Peters, L.L., Hoefer, M.T. Dopamine in hypophysial portal blood: relationship to circulating prolactin in pregnant and lactating rats. Endocrinology 106: 690-6, 1980.

Ben-Jonathan, N., Oliver, C., Weiner, H.J., Mical, R.S., Porter, J.C. Dopamine in hypophysial portal plasma of the rat during the estrous cycle and throughout pregnancy. Endocrinology 100: 452-8, 1977.

Berghorn, K., Duska, B., Wang, H., Le, W., Sherman, T., Hoffman, G. Cellular analysis of tyrosine hydroxylase expression during lactation. Society for Neuroscience, Vol. 21, pp. 1899, 1995.

Berghorn, K.A., Le, W.W., Sherman, T.G., Hoffman, G.E. Suckling stimulus suppresses messenger RNA for tyrosine hydroxylase in arcuate neurons during lactation. J. Comp. Neurol. 438: 423-32, 2001.

DOI:10.14753/SE.2012.1755

Bisset, G. W., Clarke, B. J., Lewis, G. P. The mechanism of the inhibitory action of adrenaline on the mammary gland. Br. J. Pharmacol. Chemother. 31, 550-559, 1967.

Blank, M.S., Panerai, A.E., Friesen, H.G. Effects of naloxone on luteinizing hormone and prolactin in serum of rats. J. Endocrinol. 85: 307-15, 1980.

Bodnár, I., Bánky, Z., Zelena, D., Halász, B. Glutamate receptor antagonist infused into the hypothalamic suprachiasmatic nuclei interferes with the diurnal fluctuations in plasma prolactin and corticosterone levels and injected into the mesencephalic dorsal raphe nucleus attenuates the suckling stimulus induced release of prolactin of the rat. Brain Res. Bull. 80: 9-16, 2009.

Bodnár, I., Bánky, Zs., Tóth, B., Nagy, G.M., Halász, B. Brain structures mediating the suckling stimulus-induced release of prolactin. J. Neuroendocrinol. 14: 384-96, 2002.

Boldogkői, Z., Erdélyi, F., Fodor, I. A putative latency promoter/enhancer (P(Lat2)) region of pseudorabies virus contains a virulence determinant. J. Gen. Virol. 2: 415-420, 2000.

Carter, D.A., Lightman, S.L. Oxytocin responses to stress in lactating and hyperprolactinaemic rats. Neuroendocrinology 46: 532-537, 1987.

Cicero, T.J., Schainker, B.A., Meyer, E.R. Endogenous opioids participate in the regulation of the hypothalamus-pituitary-luteinizing hormone axis and testosterone's negative feedback control of luteinizing hormone. Endocrinology 104: 1286-1291, 1979.

Ciofi, P., Crowley,.W.R., Pillez, A., Schmued, L.L., Tramu, G., Mazzuca, M. Plasticity in expression of immunoreactivity for neuropeptide Y, enkephalins and neurotensin in the hypothalamic tubero-infundibular dopaminergic system during lactation in mice. J. Neuroendocrinol. 5: 599-602,1993.

Coolen, L., Veenig, J., Peterson, D. and Shipley, M. Parvocellular subparafascicular thalamic nucleus in the rat: anatomical and functional compartmentalization. J. Comp. Neurol. 2:117-131, 2003.

Coolen, L.M., Peters, H.J. Veening, J.G. Fos immunoreactivity in the rat brain following consummatory elements of sexual behavior: a sex comparison. Brain Res. 738: 67-82, 1996.

Cosman, D., Lyman, S.D., Idzerda, R.L., Beckmann, M.P., Park, L.S., Goodwin, R.G., March, C.J. A new cytokine receptor superfamily. Trends Biochem. Sci. 15: 265-270, 1990.

De Bellis, A., Bizzarro, A., Pivonello, R., Lombardi, G., Bellastella, A. Prolactin and autoimmunity. Pituitary 8: 25-30, 2005.

de Greef, W.J., Plotsky, P.M. Neill, J.D. Dopamine levels in hypophysial stalk plasma and prolactin levels in peripheral plasma of the lactating rat: effects of a simulated suckling stimulus. Neuroendocrinology 32: 229-33, 1981.

Demarest, K.T., McKay, D.W., Riegle, G.D., Moore, K.E. Biochemical indices of tuberoinfundibular dopaminergic neuronal activity during lactation: a lack of response to prolactin. Neuroendocrinology 36: 130-7, 1983.

Dewey, K.G. Maternal and fetal stress are associated with impared lactogenesis in humans. J. Nutr. 131: 30125-30155, 2001.

Di Mattia, G.E., Gellersen, B., Bohnet, H.G., Friesen, H.G. A human B-lymphoblastoid cell produces prolactin. Endocrinology 122: 2508-17, 1988.

Dobolyi, A., Palkovits, M., Usdin, T.B. Expression and distribution of tuberoinfundibular peptide of 39 residues in rat central nervous system. J. Comp. Neurol. 455: 547-66, 2003.

Drouva, S.V., Epelbaum, J., Tapia-Arancibia, L., Laplante, E., Kordon, C. Opiate receptors modulate LHRH and SRIF release from mediobasal hypothalamic neurons. Neuroendocrinology 32: 163-7, 1981.

Dubois-Dauphin, M., Armstrong, W.E., Tribollet, E., Dreifuss, J.J. Somatosensory systems and the milk-ejection reflex in the rat. II. The effects of lesions in the ventroposterior thalamic complex, dorsal columns and lateral cervical nucleus-dorsolateral funiculus. Neuroscience 15: 1131-1140, 1985.

Dubois-Dauphin, M., Armstrong, W.E., Tribollet, E., Dreifuss, J.J. Somatosensory systems and the milk-ejection reflex in the rat. II. The effects of lesions in the ventroposterior thalamic complex, dorsal columns and lateral cervical nucleus-dorsolateral funiculus. Neuroscience 15:1131-40, 1985.

Dürr, K., Gömüc, B., Suarez, E., Hannibal, J., Meister, B. Presence of pituitary adenylate cyclase-activating polypeptide (PACAP) defines a subpopulation of hypothalamic POMC neurons. Brain Res. 1186: 203-211, 2007.

Erdmann, S., Ricken, A., Merkwitz, C., Struman, I., Castino, R., Hummitzsch, K., Gaunitz, F., Isidoro, C., Martial, J., Spanel-Borowski, K. The expression of prolactin and its cathepsin D-mediated cleavage in the bovine corpus luteum vary with estrous cycle. Am. J. Physiol. Endocrinol. Metab. 293: E1365-1377, 2007.

Everett, J.W. Functional corpora lutea maintained for months by autografts of rat hypophysis. Endocrinology 58: 786-796, 1956.

Everett, J.W. Luteotropic function of autografts of the rat hypophysis. Endocrinology 54: 685-690, 1954.

Eyal, O., Jomain, J.-B., Kessler, C., Goffin, V., Handwerger, S. Autocrine prolactin inhibits human uterine decidualization: a novel role for prolactin. Biol. Reprod. 76: 777-83, 2007.

Factor, E.M., Mayer, A.D. and Rosenblatt, J.S. Peripeduncular nucleus lesions in the rat: I. Effects on maternal aggression, lactation, and maternal behavior during pre- and postpartum periods. Behav. Neurosci. 107: 166-85, 1993.

Factor, E.M., Mayer, A.D. Rosenblatt, J.S., Peripeduncular nucleus lesions in the rat: I. Effects on maternal aggression, lactation, and maternal behavior during pre- and postpartum periods. Behav. Neurosci. 107: 166-85, 1993.

Fahie-Wilson, M.N., John, R., Ellis, A.R. Macroprolactin: high molecular mass forms of circulating prolactin. Ann. Clin. Biochem. 42: 175-192, 2005.

Febo, M., Stolberg, T.L., Numan, M., Bridges, R.S., Kulkarni, P., Ferris, C.F. Nursing stimulus is more than tactile sensation: it is a multisensory experience. Homon behav. 54: 330-9, 2008.

Ferrara, N., Clapp, C., Weiner, R. The 16K fragment of prolactin specifically inhibits basal or fibroblast growth factor stimulated growth of capillary endothelial cells. Endocrinology 129: 896-900, 1991.

Findlay, A.L.R., Grosvenor, C.E. The role of mammary gland innervation in the control of the motor apparatus of the mammary gland: a review. Physiol. rev. Dairy Sci. Abstr. 31: 109-116, 1969.

Fitzsimmons, M., Olschowka, J., Wiegand, S. Hoffman, G.E. Interaction of opioid peptide-containing-terminals with dopaminergic perikarya in the rat hypothalamus, Brain Research 581: 10-18, 1992.

Franke-Radowiecka, A. Distribution of neurons supplying the porcine mammary gland. Anat. Histol. Embryol. 36: 139-146, 2007.

Frawley, L.H., Boockfor, F.R. Mammosomatotropes: Presence and functions in normal and neoplastic pituitary tissue. Endocrine Rev. 12: 337-355, 1991.

Freeman, M.E., Kanyicska, B., Leranth, A., Nagy, G.M. Prolactin: Structure, function, and regulation of secretion. Physiol. Reviews 80: 1523-1631, 2000.

Fuxe, K., Hokfelt, T. On the existence of dopaminergic and noradrenergic mechanisms in the control of hormone releasing and inhibitory factor secretion from the median eminence. J. Anat. 111: 475-6, 1972.

Fuxe, K., Hökfelt, T., Eneroth, P., Gustafsson, J.A., Skett, P. Prolactin-like immunoreactivity: localization in nerve terminals of rat hypothalamus. Science 196: 899-900, 1977.

Gerendai, I., Tóth, I.E., Kocsis, K., Boldogkői, Z., Medveczky, I., Halász, B. Transneuronal labelling of nerve cells in the CNS of female rat from the mammary gland by viral tracing technique. Neuroscience 108: 103-118, 2001.

Goffin, V., Martial, J.A., Summers, N.L. Use of a model to understand prolactin and growth hormone specificities. Protein Engineering 8: 1215-1230, 1995.

Golander, A., Hurley, T., Barrett, J., Hizi, A., Handwerger, S. Prolactin synthesis by human chorion-decidual tissue: Possible source of prolactin in amniotic fluid. Science 202: 311-313, 1978.

Gonzalez-Martinez, T., Perez-Pinera, P., Diaz-Esnal, B., Vega, J.A. S-100 proteins in human peripheral nervous system. Microsc. Res. Tech. 15: 633-638, 2003.

Gosselin, R.E., Blankstein, J., Dent, D.W., Hobson, W.C., Fuller, G.B., Reyes, F.I., Winter, J.S., Faiman, C. Effects of naloxone and an enkephalin analog on serum prolactin, cortisol, and gonadotropins in the chimpanzee. Endocrinology 112: 2168-73, 1983.

Grandi, D., Campanini, N., Becchi, G., Lazzaretti, M. On the myoepithelium of human salivary glands. An immunohistochemical study. Eur. J. Morphol 38: 249-255, 2000.

Grossman, A., Moult, P.J., Gaillard, R.C., Delitala, G., Toff, W.D., Rees, L.H., Besser, G.M. The opioid control of LH and FSH release: effects of a met-enkephalin analogue and naloxone. Clin. Endocrinol. (Oxf) 14: 41-7, 1981.

Grossman, A., Moult, P.J., Gaillard, R.C., Delitala, G., Toff, W.D., Rees, L.H., Besser, G.M. The opioid control of LH and FSH release: effects of a met-enkephalin analogue and naloxone. Clin. Endocrinol. (Oxf) 14: 41-7, 1981.

Grosvenor, C.E., Mena, F., Whitworth, N.S. The secretion rate of prolactin in the rat during suckling and its metabolic clearance rate after increasing intervals of nonsuckling. Endocrinology 104: 372-6, 1979.

Grundmann, S.J., Pankey, E.A., Cook, M.M., Wood, A.L., Rollins, B.L., King, B.M. Combination unilateral amygdaloid and ventromedial hypothalamic lesions: evidence for a feeding pathway. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288: R702-7, 2005.

Guillaumot, P., Benahmed, M. Prolactin receptors are expressed and hormonally regulated in rat Sertoli cells. Mol. Cell Endocrinol 149: 163-168, 1999.

Handwerger, S., Richards, R.G., Markoff, E. The physiology of decidual prolactin and other decidual protein hormones. Trends Endocrinol. Metab. 3: 91-95, 1990.

Hansen, S., Kohler, C. The importance of the peripeduncular nucleus in the neuroendocrine control of sexual behavior and milk ejection in the rat, Neuroendocrinology 39: 563-72, 1984.

Hanwell, A., Linzell, J.L. The effects of engorgement with milk and of suckling on mammary blood flow in the rat. J. Physiol. 233: 111-125, 1973

Harlan, R.E., Shivers, B.D., Fox, S.R., Kaplove, K.A., Achachter, B.S., Pfaff, D.W. Distribution and partial characterization of immunoreactive prolactin in the rat brain. Neuroendocrinology 49: 7-22, 1989.

Harper, M.E., Sibley, P.E.C., Pieling, W.B., Griffiths, K. The prostatic cell: structure and function. Alan R. Liss Inc. New York. 1981, pp 115-218.

Higuchi, T., Kawakami, M. Naltrexone-induced LH release in ovariectomized estrogen-primed rats. Endocrinol. Jpn. 28: 45-50, 1981.

Ho, T.W.C., Greenan, J.R., Walker, A.M. Mammotroph autoregulation: the differential roles of the 24K isoforms of prolactin. Endocrinology 124: 1507-1514, 1989.

Hoffman, G.E., Le, W.W. Just cool it! Cryoprotectant anti-freeze in immunocytochemistry and in situ hybridization. Peptides 25, 425-31, 2004.

Hondo, E., Kurohmaru, M., Sakai, S., Ogawa, K., Hayashi, Y. Prolactin receptor expression in rat spermatogenic cells. Biol. Reprod. 52: 1284-1290, 1995.

Horseman, N.D., Zhao, W., Montecino-Rodriguez, E., Tanaka, M., Nakashima, K., Engle, S.J., Smith, F., Markoff, E., Dorshkind, K. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. EMBO J. 16: 6926-6935, 1997.

Imaoka, T., Matsuda, M., Mori, T. Expression of prolactin messenger ribonucleic acid in the mouse gonads during sexual maturation. Life Sci. 63: 2251-2258, 1998.

Jakab, G., Salamon, I., Petrusz, P., Réthelyi, M. Termination patterns of calcitonin gene-related peptide-immunoreactive nerve fibers in the dorsal horn of the human spinal cord. Exp. Brain Res. 80: 609-617,1990.

Kehoe, L., Janik, J., Callahan, P. Effects of immobilization stress on tuberoinfundibular dopaminergic (TIDA) neuronal activity and prolactin levels in lactating and non-lactating female rats. Life Sci. 50, 55-63, 1992.

Kelly, P.A., Ali, S., Rozakis, M., Goujon, L., Nagano, M., Pellegrini, I., Gould, D., Djiane, J., Edery, M., Finidori, J., Postel-Vinay, M.C. The growth hormone/prolactin receptor family. Recent Prog. Horm. Res. 48: 123-164, 1993.

Koizumi, M., Horiguchi, K., Tomitam Y., Kato, Y., Harigaya, T. Prolactin gene expression in the mouse nipple. J. Reprod. Dev. 49: 465-72, 2003.

Köves, K., Heinzlmann, A. Neurotransmitters and Neuropeptides in Autism. In: New Autism Research Developments. Ed. Mesmere B., Nova Science Publishers, Inc., New York, 2007, pp. 25-85.

Kurtz, A., Bristol, L.A., Tóth, B.E., Lazar-Wesley, E., Takács, L., Kacsóh, B. Mammary epithelial cells of lactating rats express prolactin messenger ribonucleic acid. Biol. Reprod. 48: 1095-1103, 1993.

Landis, S.C, Fredieu, J.R Coexistence of calcitonin gene-related peptide and vasoactive intestinal peptide in cholinergic sympathetic innervation of rat sweat gland. Brain Res. 377, 177-181, 1986.

Ledoux, J.E., Ruggiero, D.A., Forest, R., Stornetta, R., Reis, D.J. Topographic organization of convergent projections to the thalamus from the inferior colliculus and spinal cord in the rat. J. Comp. Neurol . 264: 123-46, 1987.

Leong, D.A., Frawley, L.S., Neill, J.D. Neuroendocrine control of prolactin secretion. Ann. Rev. Physiol. 45: 109-27, 1983.

Li, C., Chen, P., Smith, M.S. Identification of neuronal input to the arcuate nucleus (ARH) activated during lactation: implications in the activation of neuropeptide Y neurons. Brain Research 824: 267-276, 1999a.

Li, C., Chen, P., Smith, M.S. Neural populations in the rat forebrain and brain stem activated by the suckling stimulus as demonstrated by c-Fos expression. Neuroscience 94: 117-129, 1999b.

Macleod, R.M., Lehmeyer, J.E. Studies on the mechanism of the dopamine-mediated inhibition of prolactin secretion. Endocrinology 94: 1077-85, 1974.

Maolood, N., Meister, B. Dynorphin in pro-opiomelanocortin neurons of hypothalamic arcuate nucleus. Neuroscience 154 154: 1121-1131, 2008.

Masler, I.A., Riddick, D.H. Prolactin production by human endometrium during the normal menstrual cycle. Am. J. Obstet. Gynecol. 135: 751-754, 1979.

May, P.B., Mittler, J.C., Ertel, N.H. Enkephalins and pituitary hormone release: modification of responsiveness to LHRH. Horm. Res. 10: 57-63, 1979.

McVea, K.L., Turner, P.D., Peppler, D.K. The role of breastfeeding in sudden infant death syndrome. J. Hum. Lact. 16: 13-20, 2000.

Meister, B., Hökfelt, T. Peptide- and transmitter-containing neurons in the mediobasal hypothalamus and their relation to GABAergic systems: possible roles in control of prolactin and growth hormone secretion. Synapse 2: 585-605, 1988.

Mena, F., Aguayo, D., Pacheco, P., Morales, M.T. Central effects of catecholamines upon mammary contractility in rat are neurally mediated. Neuroendocrinology 61: 722-730, 1995.

Mena, F., Enjalbert, A., Carbonell, L., Priam, M., Kordan, C. Effect of suckling on plasma prolactin and hypothalamic monoamine levels in the rat. Endocrinology 99: 445-51, 1976.

Mena, F., Pacheco, P., Aguayo, D., Martinezm G., Grosvenorm C.E. Reflex regulation of autonomic influences upon the oxytocin-induced contractile response of mammary gland in the anesthetized rat. Endocrinology 104: 751-756,1979.

Merchenthaler, I. Induction of enkephalin in tuberoinfundibular dopaminergic neurons of pregnant, pseudopregnant, lactating and aged female rats. Neuroendocrinology 60: 185-93, 1994.

Merchenthaler, I. Induction of enkephalin in tuberoinfundibular dopaminergic neurons during lactation. Endocrinology 133: 2645-51, 1993.

Merchenthaler, I., Lennard, D.E., Cianchetta, P., Merchenthaler, A., Bronstein, D. Induction of proenkephalin in tuberoinfundibular dopaminergic neurons by hyperprolactinemia: the role of sex steroids. Endocrinology 136: 2442-50, 1995.

Meuris, S., Verloes, A., Robyn, C. Immunocytochemical localization of prolactin-like immunoreactivity in rat pancreatic islets. Endocrinology 112: 2221-2223, 1983.

Michaloudi, H.C., el Majdoubi, M., Poulain, D.A., Papodopoulos, G.C., Theodosis, D.T. The noradrenergic innervation of identified hypothalamic magnocellular somata and its contribution to lactation-induced synaptic plasticity. J Neuroendocrinol 9: 17-23, 1997.

Montgomery, D.W., Shen, G.K., Ulrich, E.D., Steiner, L.L., Parrish, P.R., Zukoski, C.F. Human thymocytes express a prolactin-like messenger ribonucleic acid and synthesize bioactive prolactin-like proteins. Endocrinology 131: 3019-3026, 1992.

Morales, T., Shapiro, E., Marina, N., Mena, F. Sympathetic innervation of mammary gland mediates suckling-induced reflex inhibition of milk yield in rats. Physiol. Behav. 74: 37-43, 2001.

Morel, G., Ouhtit, A., Kelly, P.A. Prolactin receptor immunoreactivity in rat anterior pituitary. Neuroendocrinology 59: 78-84, 1994.

Motta, M., Martini, L. Effect of opioid peptides on gonadotrophin secretion. Acta Endocrinol. (Copenh) 99: 321-5, 1982.

Moult, P.J., Grossman, A., Evans, J.M., Rees, L.H., Besser, G.M. The effect of naloxone on pulsatile gonadotrophin release in normal subjects. Clin. Endocrinol. (Oxf) 14: 321-4, 1981.

Nagano, M., Kelly, P.A. Tissue distribution and regulation of rat prolactin receptor gene expression. J. Biol. Chem. 269: 13337-13345, 1994

Neill, J.D., Nagy G.M. Prolactin secretion and its control. In: Knobil, E, Neill, J.D. The physiology of reproduction, Raven Press, New York,1994, pp. 1833-1860.

Nevalainen, M.T., Valve, E.M., Ahonen, T., Yagi, A., Paranko, J., Härkönen, P.L. Androgen dependent expression of prolactin in rat prostate epithelium in vivo and in organ culture. FASEB J. 11: 1297-1307, 1997.

Nicoll, C.S., Mayer, G.L., Russell, S.M. Stuctural features of prolactins and growth hormones that can be related to their biological properties. Endocr. Rev. 7:169-203, 1986.

Nolin, J.M. Intracellular prolactin in rat corpus luteum and adrenal cortex. Endocrinology 102: 402-406, 1978.

Nowak, R.A., Rein, M.S., Heffner, L.J., Friedman, A.J., Tashjian Jr, A.H. Production of prolactin by smooth muscle cells cultured from human uterine fibroid tumors. J. Clin. Endocrinol. Metab. 76: 1308-1313, 1993.

Oláh, M., Fehér, P., Ihm, Z., Bácskay, I., Kiss, T., Freeman, M.E., Nagy, G.M., Vecsernyés, M. Dopamine-regulated adrenocorticotropic hormone secretion in lactating rats: functional plasticity of melanotropes. Neuroendocrinology 90, 391-401 2009.

Parlow, A.F., Shome, B. Rat prolactin: the entire linear amino acid sequence. Fed. Proc. 35: 219, 1976.

Paut-Pagano, L., Roky, R., Valatx, J.L., Kitahama, K., Jouvet, M. Anatomical distribution of prolactin-like immunoreactivity in the rat brain. Neuroendocrinology 58: 682-695, 1993.

Paxinos, G., Watson, C. The rat brain in stereotaxic coordinates. Academic Press, San Diego, 1986.

Pellegrini, I., Lebrun, J.J., Ali, S., Kelly, P.A. Expression of prolactin and its receptor in human lymphoid cells. Mol. Endocrinol. 6: 1023-31, 1992.

Pena, K.S., Rosenfeld, J.A., Evaluation and treatment of galactorrhea. Am. Farm Physician 63: 1763-70, 2001.

Pimpinelli, F., Parenti, M., Guzzi, F., Piva, F., Hökfelt, T., Maggi, R. Presence of delta opioid receptors on a subset of hypothalamic gonadotropin releasing hormone (GnRH) neurons. Brain Res. 1070: 15-23, 2006.

Pinho, M.S., Gubelkian, S. Innervation of the canine mammary gland: an immunohistochemical study. Histo. Histopathol. 22, 1175-1184, 2007.

Plotsky, P.M., De Greef, W.J. Neill, J.D. In situ voltametric microelectrodes: application to the measurement of median eminence catecholamine release during simulated suckling. Brain Res 250: 251-62, 1982.

Plotsky, P.M., Neill, J.D. The decrease in hypothalamic dopamine secretion induced by suckling: comparison of voltammetric and radioisotopic methods of measurement. Endocrinology 110: 691-6, 1982.

Riddle, O., Bates, R.W., Dykshorn, S.W. A new hormone of the anterior pituitary. Proc. Soc. Exp. Biol. Med. 29: 1211-1215, 1932.

Robertson, M.T., Alho, H.R., Martin, A.A. Localization of prolactin-like immunoreactivity in grafted human sweat glands. J. Histochem. Cytochem. 37: 625-8, 1989.

Roky, R., Paut-Pagano, L., Goffin, V., Kitahama, K., Valatx, Kelly, P.A., Jouvet, M. Distribution of prolactin receptors in the rat forebrain. Immunohistochemical study. Neuroendocrinology 63: 422-29, 1996.

Rosenberg, S.M., Masler, I.A., Riddick, D.H. Decidual production of prolactin in late gestation: further evidence for a decidual source of amniotic fluid prolactin. Am. J. Obstet. Gynecol. 138: 681-685, 1980.

Roux, M., Martinat, N., Richoux, J-R., Grignon, G. Histoimmunological identification of a prolactin-like substance in rodent testes. Cell Tissue Res. 240: 663-667, 1985.

Samson, W.K., Lumpkin, M.D., McCann, S.M. Evidence for a physiological role for oxytocin in the control of prolactin secretion. Endocrinology 119: 554-560, 1986.

Sanders, G., Freilicher, J., Lightman, S.L. Psychological stress of exposure to uncontrollable noise increases plasma oxytocin in high emotionality women. Psychoneuroendocrinology 15: 40-54, 1990.

Schäfer, M.K., Schütz, B., Weihe, E., Eiden, L.E. Target independent cholinergic differentiation in the rat sympathetic nervous system. Proc. Natl. Acad. Sci. USA 94, 4149-4154, 1997.

Schillo, K.K., Kuehl, D., Jackson, G.L. Do endogenous opioid peptides mediate the effects of photoperiod on release of luteinizing hormone and prolactin in ovariectomized ewes? Biol. Reprod. 32: 779-87, 1985.

Selmanoff, M., Wise, P.M. Decreased dopamine turnover in the median eminence in response to suckling in the lactating rat. Brain Res. 212: 101-15, 1981.

Semeniken, K., Merchenthaler, I., Hu, W., Dudas, B. Catecholaminergic input to the oxytocin neurosecretory system in the human hypothalamus. J. Chem. Neuroanat. 37: 229-233, 2009.

Simerly, R.B., Young, B.J. Regulation of estrogen receptor messenger ribonucleic acid in rat hypothalamus by sex steroid hormones. Mol. Endocrinol. 5: 424-432, 1991.

Sinha, Y. N. Structural variants of prolactin: occurrence and physiological significance. Endocrine Rev. 16: 354-369, 1995.

Slama, A., Burg-Poveda, D. Tramu, G. Colocalized peptides in gonadotrophs: LeuEnkephalin and ACTH interact differently on GnRH induced LH and FSH release. Neuropeptides 16: 135-40, 1990.

Song, S.L., Crowley, W.R., Grosvenor, C.E. Evidence for the involvement of an adrenal catecholamine in the β-adrenergic inhibition of oxytocin release in lactating rats. Brain Res. 457: 303-309, 1988.

Stricker, P., Grueter, R. Action du lobe anterieur de l'hypophyse sur la montee laiteuse. C. R. Soc. Biol. (Paris) 99: 1978-1980, 1928.

Talwalker, P.K., Ratner, A., Meites, J. *In vitro* inhibition of pituitary prolactin synthesis and release by hypothalamic extracts. Am. J. Physiol. 205: 213-218, 1963.

Tanaka, M., Suzuki, M., Kawana, T., Segawa, M., Yoshikawa, M., Mori, M., Kobayashi, M., Nakai, N., Saito, T.R. Differential effects of sex steroid hormones on the expression of multiple first exon of prolactin receptor gene in the rat liver. J. Mol. Endocrinol. 34: 667-673, 2005.

Tindal, J.S., Knaggs, G.S. An ascending pathway for release of prolactin in the brain of the rabbit. J. Endocrinol. 45: 111-20, 1969.

Tindal, J.S., Knaggs, G.S. Further studies on the afferent path of the milk-ejection reflex in the brain stem of the rabbit, J. Endocrinol. 66: 107-13, 1975.

Tindal, J.S., Knaggs, G.S., Turvey, A. The afferent path of the milk-ejection reflex in the brain of the rabbit. J. Endocrinol. 43: 663-71, 1969.

Tóth, B.E., Bodnár, I., Homicskó, K.G., Fülöp, F., Fekete, M.I.K., Nagy, G.M. Physiological role of salsolinol: Its hypophysiotrophic function in the regulation of pituitary prolactin secretion. Neurotoxicology and Teratology 24: 655-666, 2002.

Tsubura, A., Mori, S., Mori, T., Nagasawa, H. Immunoreactive PRL in mouse urethral glands. Acta Anat. (Basel) 126: 263-265, 1986.

Tucker, H.A. Neuroendocrine regulation of lactation and milk ejection. In: Neuroendocrinology in Physiology and Medicine, 2000. (Eds.: P.M. Conn and M.E.Freeman) pp. 163-180.

Tucker, H.A. Physiological control of mammary growth, lactogenesis, and lactation. J. Dairy Sci. 64: 1403-1421, 1981.

Untergasser, G., Kranewitter, W., Walser, F., Madersbacher, S., Dirnhofer, S., Berger, P. The testis as eutopic production site of human growth hormone, placental lactogen and prolactin: possible autocrine/paracrine effects on testicular function. Wien Klin. Wochenschr. 108: 541-546, 1996.

Untergasser, G., Rumpold, H., Plas, E., Witkowski, M., Berger, P. Seminal plasma factors induce in vitro PRL secretion in smooth muscle cells of the human prostate. J. Clin. Endocrinol. Metab. 86: 5577-5584, 2001.

Usdin, T.B., Hoare, S.R., Wang, T., Mezey, E., Kowalak, J.A. TIP39: a new neuropeptide and PTH2-receptor agonist from hypothalamus. Nat. Neurosci. 2: 941-3, 1999.

Van Vugt, D.A., Bakst, G., Dyrenfurth, I., Ferin, M. Naloxone stimulation of luteinizing hormone secretion in the female monkey: influence of endocrine and experimental conditions. Endocrinology 113: 1858-64, 1983.

Van Vugt, D.A., Lam, N.Y., Ferin, M. Reduced frequency of pulsatile luteinizing hormone secretion in the luteal phase of the rhesus monkey. Involvement of endogenous opiates. Endocrinology 115: 1095-101, 1984.

Veening, J.G., Coolen, L.M. Neural activation following sexual behavior in the male and female rat brain. Behav. Brain Res. 92: 181-93, 1998.

Walker, A.M., Montgomery, D.W., Ho, T.W.C. Human serum prolactin-IgG complexes: localization of PRL to heavy chain FAB and stimulation of lymphocyte proliferation *in vitro*. Program of the 75th Annual Meeting of the Endocrine Society, Las Vegas, NV, 1993, p. 163.

Walker, A.M., Montgomery, D.W., Saraiya, S., Ho, T.W., Garewal, H.S., Wilson, J., Lorand, L. Prolactin immunoglobulin G complexes from human serum act as costimulatory ligands causing proliferation of malignant B lymphocytes. Proc. Natl. Acad. Sci. USA. 92: 3278-3282, 1995.

Walters, C.A., Daly, D.C., Chapitis, J., Kuslis, S.T., Prior, J.C., Kusmik, W.F., Riddick, D.H. Human myometrium: a new potential source of prolactin. A. J. Obstet. Gynecol. 147: 639-644, 1983.

Wang, H.J., Hoffman, G.E., Smith, M.S. Suppressed tyrosine hydroxylase gene expression in the tuberoinfundibular dopaminergic system during lactation. Endocrinology 133: 1657-63, 1993.

Wang, Y.F., Negoro, H., Honda, K. Amplitudes and time courses of milk ejection bursts of oxytocin neurons in the midbrain-hemitransected lactating rat. Brain Res. 719: 203-6, 1996a.

Wang, Y.F., Negoro, H., Honda, K. Milk ejection bursts of supraoptic oxytocin neurones during bilateral and unilateral suckling in the rat, J Neuroendocrinol, 8 427-31, 1996b.

Wang, Y.F., Negoro, H., Honda, K. Effects of hemitransection of the midbrain on milk-ejection burst of oxytocin neurones in lactating rat. J. Endocrinol. 144: 463-70, 1995.

Watson, R., Wiegand, S., Clough, R., Hoffman, G.E. Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. Peptides 7, 155-159, 1986.

Whitworth, N.S., Grosvenor, C.E. The effect of exteroceptive pup stimuli on the responsiveness of prolactin release mechanisms to suckling stimuli in the lactating rat. Endocrinology, 115: 1135-40, 1984.

Wilson, D.M.3rd, Emanuele, N.V., Jurgens, J.K., Kelley, M.R. Prolactin message in brain and pituitary of adult male rats is identical: PCR cloning and sequencing of hypothalamic prolactin cDNA from intact and hypophysectomized adult male rats. Endocrinology 131: 2488-90, 1992.

Wood, R.L., Zhang, J., Huang, Z.M., Gierow, J.P., Schechter, J.E., Mircheff, A.K., Warren, D.W. Prolactin and prolactin receptors in the lacrimal gland. Exp. Eye Res. 69: 213-226, 1999.

Yasui, Y., Saper, C.B., Cechetto, D.F. Calcitonin gene-related peptide immunoreactivity in the visceral sensory cortex, thalamus, and related pathways in the rat. J. Comp. Neurol. 290: 487-501, 1989.

Youngren, O., Yupaporn, C., Khaled, A-Z., Whiting, S., Kang, S.W., El Halavani, M. Regulation of prolactin secretion by dopamine at the level of the hypothalamus in the turkey. Neuroendocrinology 75: 185-192, 2002.

Zhang, F.P., Rannikko, A., Toppari, J., Bartke, A., Huhtaniemi, I. Developmental expression of the prolactin receptor gene in rat gonads. J. Endocrinol. 147: 497-505, 1995.

DOI:10.14753/SE.2012.1755

12. LIST OF PUBLICATIONS

PUBLICATIONS RELATED TO THE THESIS

Szabo, F. K., Snyder, N., Usdin, T. B., Hoffman, G. E. A direct neuronal connection between the subparafascicular and ventrolateral arcuate nuclei in non-lactating female rats. Could this pathway play a role in the suckling-induced prolactin release? Endocrine 37: 62-70, 2010 (IF 1.342)

Szabo, F. K., Le, W. W., Snyder N., Hoffman, G. E. Comparison of the temporal programs regulating thyrosin hydroxylase and enkephalin expressions in TIDA neurons of lactating rats following pup removal and then pup return. J. Mol. Neurosci. 45: 110-118, 2011 (IF 2.922)

Köves, K., Györgyi, Z., **Szabó, F. K.,** Boldogkői, Z. Chemical characterization of the autonomic innervation of the nipple and the mammary gland of lactating rats studied by retrograde trans-synaptic virus labeling and immunohistochemistry. Acta Physiol. Hung. (accepted, 2012) (IF 1.227)

PUBLICATIONS NOT RELATED TO THE THESIS

Szabó, F., Horváth, J., Heinzlmann, A., Arimura, A., Köves, K. Neonatal PACAP administration in rats delays puberty through the influence of the LHRH neuronal system. Regul. Pep. 109: 49-55, 2002 (IF 3.205).

Köves, K., Vereczki, V., Kausz, M., Kántor, O., Molnár, J., Nemeskéri, Á., Heinzlmann, A., Szabó, E., **Szabó, F**., Fógel, K., Lakatos, A., Szeiffert, G., Arimura, A. PACAP and VIP in the photoneuroendocrine system (PNES). Med. Sci. Mon. 8: SR5-20, 2002.

Köves, K., Kántor, O., Molnár, J., Heinzlmann, A., Szabó, E., **Szabó, F.,** Nemeskéri, Á., Horváth, J., Arimura, A. The role of PACAP in gonadotropic hormone secretion at hypothalamic and pituitary levels. J. Mol. Neurosci. 20: 141-152, 2003 (IF 1.735).

Szabo, F. K., Lomenick, J. P. Syndrome of inappropriate antidiuretic hormone secretion in an infant with respiratory syncytial virus bronchiolitis. Clin. Pediatr. (Phila) 47: 840-2, 2008 (IF 1.5)

DOI:10.14753/SE.2012.1755

13. ACKNOWLEDGEMENT

I would like to thank Professor Gloria Hoffman for the opportunity to work in her laboratory under her guidance toward compiling this work. Many thanks for Natelie Snyder who helped me with all the technical work. Her strong work ethics and precision was crucial in gathering data. I am also grateful to WeiWei Le for always being there with technical help.

Lots of thanks to my fellow PhD student at the time, Takisha Shultebrandt for being my friend and moral support.

And most of all gratitude I cannot express to my mentor, Professor Katalin Köves, who wanted nothing more than for me to succeed and grow and gave her time, expertise and energy unselfishly throughout these years.