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ORIGINAL ARTICLE

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?

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Abstract The neuronal pathways, through which prolactin secretion is regulated during lactation, have still not been fully explored. Studies indicate that the suckling stimulus travels through the spinal cord, the brain stem, and then reaches the hypothalamus. The focus of this present experiment is to further explore the neuronal connections between the brain stem and the arcuate nucleus that may 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. 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 in the pathway involved in the suckling-induced prolactin release.

Keywords Rat · Lactation · Prolactin · Ante- and retrograde tracing · Immunohistochemistry

Abbreviations

ARC	Arcuate nucleus
BDA	Biotinylated dextran amine
CGRP	Calcitonin gene-related peptide
DAB	Diaminobenzidine tetrahydrochloride
Dyn	Dynorphine
FG	Fluorogold
Gal	Galanine
KPBS	Potassium-phosphate buffer saline
NKB	Neurokinin-B
OT	Oxytocin
PFA	Paraformaldehyde
PPN	Peripeduncular nucleus
PRL	Prolactin
PV	Paraventricular nucleus
SPFpc	Subparafascicular parvocellular nucleus

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TH	Tyrosine hydroxylase
TIDA neurons	Tuberoinfundibular dopaminergic neurons
TIP39	Tuberoinfundibular peptide of 39 residues
VMN	Ventromedial nucleus

Introduction

In the past years, a lot of attention has been focused on the importance of suckling in the success of lactation. The most widely studied neuroendocrine reflex responsible for milk production is the suckling-induced prolactin (PRL) release. It is well established that PRL secretion and release by mammotropes in lactating rats is mainly controlled by dopaminergic neurons of the medial basal hypothalamus [1]. Dopamine has been identified as the main inhibitory transmitter, responsible for tonic inhibition of PRL production and release in non-lactating rats. At the beginning of lactation, suckling stimuli reach the hypothalamus. These stimuli inhibit the activity of the tuberoinfundibular dopaminergic (TIDA) neurons located in the A12 catecholaminergic cell group of the arcuate nucleus (ARC) [2]. Decreasing dopamine release allows the release of PRL from the pituitary into the general circulation and in turn, PRL stimulates milk secretion.

Suckling also stimulates oxytocin (OT) release from the magnocellular supraoptico-paraventriculo-hypophyseal system. Previous reports have suggested that the release of PRL and OT during suckling are coordinated [3, 4]. A profile of brain sites involved in the suckling-induced neuroendocrine axis has emerged by monitoring milk letdown reflexes as a result of OT release or electrical activity of identified OT neurons after brain stimulation or following suckling after lesions. Studies indicate that the suckling stimulus from the mechanoreceptors of the nipples is delivered to the spinal cord and relayed in the cervical spinal nucleus [5]. After ascending from this nucleus, a projection to the most cranial part of the mesencephalic tegmentum [5–8] rather than the more classical thalamic sites [5], conveys suckling signals to the hypothalamus for milk ejection control.

The specific site, where signals regulating PRL or OT are processed, is not well defined. There appears to be at least one additional relay before the TIDA 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 success of lactation. Such observations were made based on studies in which this area was lesioned [6, 9] and lactation was impaired. Experiments using stimulation paradigms noted that the PPN [8, 10] was effective in releasing PRL. It was also observed that electrical stimulation of the same area (rostromedial parts of the midbrain tegmentum) also induced milk ejection [11]. An interneuronal relay from the mesencephalon to the hypothalamus was proposed, but has yet to be identified for either projection to the OT or TIDA system. Since the suckling stimulus excites OT 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.

A retrograde tracing study where a rather large fluorogold (FG) injection was applied into the ARC, spreading beyond the arcuate borders to the adjacent ventromedial complex, indicated that the mesencephalic PPN and a region medial to it contained a considerable number of retrogradely FG labeled neurons. According to stereotaxic atlases, this region medial to PPN was the most posterior part of the subparafascicular parvocellular nucleus (SPFpc) belonging to the posterior thalamus. A subpopulation of the labeled neurons was also activated by the suckling stimulus. This study verified the existence of the pathway between the hypothalamus and the midbrain [12]. The authors of the above-mentioned article did not differentiate between the PPN and SPFpc.

To resolve whether the PPN or the SPFpc provides a direct connection to the ARC itself, we conducted a series of ante- and retrograde tracing studies in non-lactating rats. FG was administered to the ARC and labeled cells were looked for in the PPN and in the neighboring posterior part of the SPFpc. Biotinylated dextran amine (BDA) was administered in those regions where FG cell bodies were observed and BDA labeled fibers were looked for in the ARC and its neighboring regions. Brain stem sections (including the posterior thalamus), containing retrogradely FG labeled neurons from the ARC, were stained for tuberoinfundibular peptide of 39 residues (TIP39), calcitonin gene-related peptides (CGRP), or galanine (Gal) immunoreactivities to clarify the kinds of peptides synthesized in these neurons. Hypothalamic sections showing BDA labeling were stained for dynorphine (Dyn) immunoreactivity. Dyn–tyrosine hydroxylase (TH) double staining was also done to demonstrate that the Dyn containing neurons reach the TIDA neurons. TIP39, CGRP, and Gal were chosen for double labeling because according to previous observations [13, 14] these peptides had been described in those midbrain neurons that were retrogradely labeled from ARC in our material.

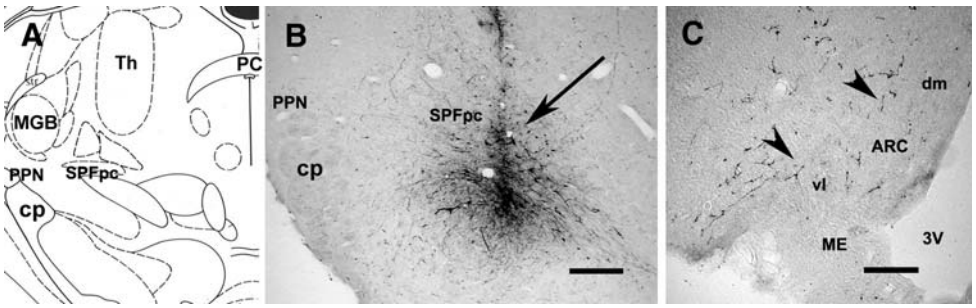

Fig. 1 The site of the BDA injection in the mesencephalon and ascending BDA fibers in the hypothalamus. **a** Schematic illustration of transsection of the mesencephalon at A 4.2 mm to the interaural line according to the Paxinos and Watson stereotaxic atlas [15]. **b** Microphotograph of a transection of the mesencephalon with the injection site of BDA (indicated by an *arrow*) at the same level as (**a**). The injection site includes SPFpc. **c** Microphotograph of a frontal section of the medial basal hypothalamus shows anterogradely transported BDA labeled fibers to ARC (*arrowheads*) from the mesencephalic site of the BDA injection. Abbreviations: *3V* third ventricle; *ARC* arcuate nucleus; *cp* cerebral peduncle; *dm* dorsomedial part of ARC; *ME* median eminence; *MGB* medial geniculate body; *PC* posterior commissure; *PPN* peripenpendicular nucleus; *SPFpc* subparafascicular nucleus, parvocellular part; *Th* thalamus; *vl* ventrolateral part of ARC. *Scale*: 500 μm in (**b**) and 250 μm in (**c**)

Results

Anterograde tracer (BDA) injection in the PPN

In the animals from Experiment 1, BDA administered exclusively into the PPN failed to label any fibers in the ARC (two animals); however, labeled fibers were found in the vicinity of the ARC, in the ipsilateral ventromedial hypothalamic nucleus (VMN) (not shown). Administration of the tracer ventromedial to the PPN actually did label fibers in the ipsilateral ARC (Fig. 1a, b). According to the stereotaxic rat brain atlas [15], this area was identified as SPFpc just caudal the posterior intralaminar thalamic nuclei, at the junction of the thalamus and mesencephalon (five animals). BDA labeled fibers were mostly located in lateral and ventral subdivisions of the ARC (Fig. 1c). The BDA/TH double label immunostaining revealed that the neurons in the proximity of BDA fibers were not TIDA neurons. BDA labeled axons did not contact TH containing TIDA neurons (Fig. 2a, b). However, BDA/Dyn double labeling revealed a close apposition between BDA labeled fibers and Dyn immunopositive cell bodies in the lateral portion of ARC (Fig. 2c). In turn, Dyn immunoreactive fibers climbed on TIDA neurons (Fig. 2d).

Retrograde tracer (FG) injection in the ventrolateral ARC

In the most rostral midbrain of the animals from Experiment 2, where FG administration included the antero-ventro-lateral portion of the ARC (Fig. 3a), there was labeling in an area located in the proximity of the posterior thalamus that consists of horizontally oriented cells extending from rostromedial to caudolateral direction fusing with the posterior intralaminal nucleus and the PPN. The distribution of these cells suggested that this area was SPFpc (in all five animals) (Fig. 3b). The PPN did not contain FG labeled cells.

As expected, we have found TIP39 immuoreactive neurons in the SPFpc. FG injected into the ARC labeled a part of TIP39 cells in this nucleus (Fig. 4a, b).

Calcitonin gene-related peptide immunoreactive cells were observed in both the SPFpc and the PPN. FG and CGRP double labeling revealed that a subpopulation of the cells in the SPFpc, which contain FG, also shows CGRP immunoreactivity (Fig. 4c, d).

Fluorogold and Gal double labeling showed that there was a dense fiber labeling with only a few Gal immunopositive cells in SPFpc. None of Gal immunopositive cells contained FG (not shown).

Discussion

The mesencephalon has long been suggested to mediate the suckling stimulus ascending from the nipples through the spinal cord. The significance of the brain stem in suckling-induced PRL release has recently been confirmed through bilateral deafferentation of the posterior hypothalamus, which completely blocked the PRL response [16]. A number of studies have suggested that specifically the PPN in the most rostral mesencephalon is critical for successful lactation [6, 8, 9, 11]. Retrograde tracing studies from the ARC by Li et al. [12] have identified the PPN as the relay nucleus in the midbrain to the ARC, known site of dopaminergic neurons involved in the regulation of PRL secretion. In our experiment, administration of an anterograde tracer exclusively in the PPN did not result in fiber labeling in the ARC; however, when the tracer got into a more medial region of the SPFpc, it resulted in fiber

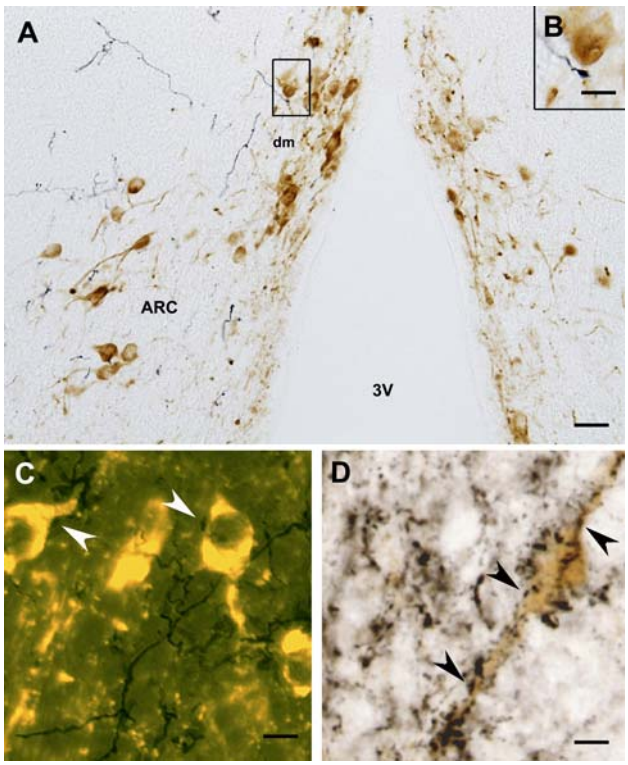

Fig. 2 Microphotographs showing double stains in the frontal sections of the medial basal hypothalamus. **a** BDA and TH double stain, TH immunopositive cells (indicated by *brown color*) located mainly in the dorsomedial part of the arcuate nucleus, ipsilateral to the site of BDA injection in the mesencephalic SPFpc, do not show any contact with BDA labeled fibers (indicated by *blue color*). Inset (**b**) shows the framed area with high magnification. **c** BDA and Dyn double stain. Dyn immunoreactive cell bodies (*orange color*) and BDA fibers (*black color*). BDA fibers exhibit close apposition on Dyn immunoreactive cell bodies and dendrites (indicated by *white arrowheads*). **d** Dyn and TH double stain. A TIDA neuron (*brown color*) is heavily innervated by Dyn fibers (*black color*). *Black arrowheads* show close contacts of the Dyn fibers and the cell body and the dendrites of the TIDA neuron. Abbreviations: *3V* third ventricle; *ARC* arcuate nucleus; *dm* dorsomedial part of ARC. Scale: 100 μm in (**a**) and 25 μm in (**b**)–(**d**)

labeling in the ARC itself. These results support a hypothesis that there is a direct neuronal connection between the SPFpc nucleus and the ventrolateral ARC. We assumed that besides the PPN, the SPFpc may also be involved in the suckling-induced PRL release. In the article by Li et al. [12], c-Fos immunoreactivity upon the stimulus of suckling can be seen not only in the PPN but in the neighboring area as well, which is identified by the stereotaxic atlas [15] as SPFpc (Fig. 4 of their article). This above-mentioned article is the evidence that the SPFpc is involved in the suckling-induced PRL release.

The VMN has been suggested to play a role in the regulation of PRL in the turkey [17]; it is unlikely that this nucleus relays suckling stimulus to TIDA neurons in mammals. However, the PPN is a very important nucleus in the process of lactation, especially since it has been shown to be activated by the suckling stimulus [12, 18] as well as by exteroceptive stimuli from pups such as visual, olfactory, and auditory in the absence of suckling. Such stimuli become especially important in late stages of lactation in regulating milk supply [19]. Unilateral chemical or radiofrequency lesioning of the PPN on post partum day 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 [6, 9]. Other studies showed that hemitransection of the midbrain tegmentum, including the region of the PPN, only blocks the milk ejection reflex from contralateral suckling [20, 21] and bilateral suckling still remains more effective than unilateral suckling in eliciting milk let-down after these lesions [22].

The results of our anterograde tracing experiments suggest the presence of 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 the 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 [12, 23]. 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.

The SPFpc is a subnucleus adjacent to the posterior intralaminar thalamus and it consists of horizontally oriented cells and extends rostromedial to caudolateral and overlies the medial lemniscus [14, 24]. 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 were not TIDA cells. Therefore, these neurons of the ARC are probably just a relay population to TIDA cells. Previous experiments suggest that about 70% of TIDA neurons are innervated by Dyn containing axons [25]. We hypothesized that the BDA labeled axons in the ventrolateral part of the ARC, originating from the SPFpc, actually terminated on Dyn neurons. BDA–Dyn double labeled immunocytochemistry did prove this assumption. It is possible that these cells are excitatory by nature and indirectly (through another set of relay neurons, for example, through the dorsomedial ARC) inhibit TIDA neurons during lactation. The exact circuit within the ARC thus still needs to be explored.

The injection of the retrograde tracer FG confined to the ARC resulted in labeled cells in the SPFpc, ventral and medial to the PPN. Fos studies show this nucleus to be

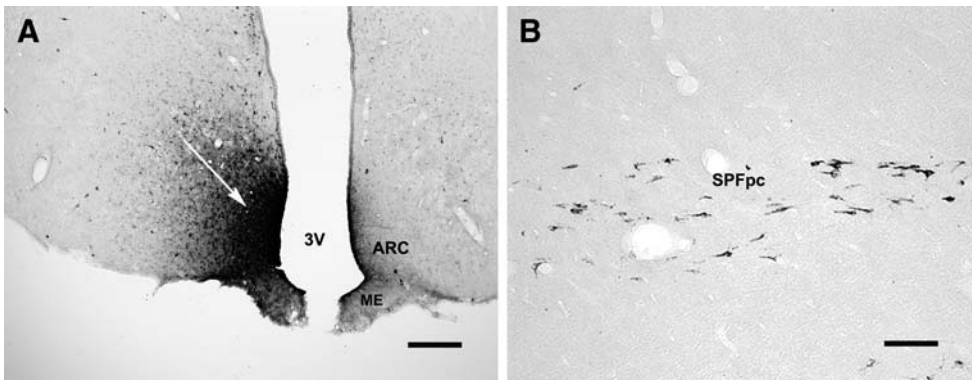

Fig. 3 Microphotographs of a frontal section of the hypothalamus and horizontal section of the mesencephalon of the same animal. **a** The site of FG injection is confined to ARC indicated by the *white arrow*. **b** The FG containing cells in the mesencephalic SPFpc are labeled in a retrograde manner. Abbreviations: *3V* third ventricle; *ARC* arcuate nucleus; *ME* median eminence; *SPFpc* parvocellular part of subparafascicular nucleus. *Scale*: 500 μm in (**a**) and 250 μm in (**b**)

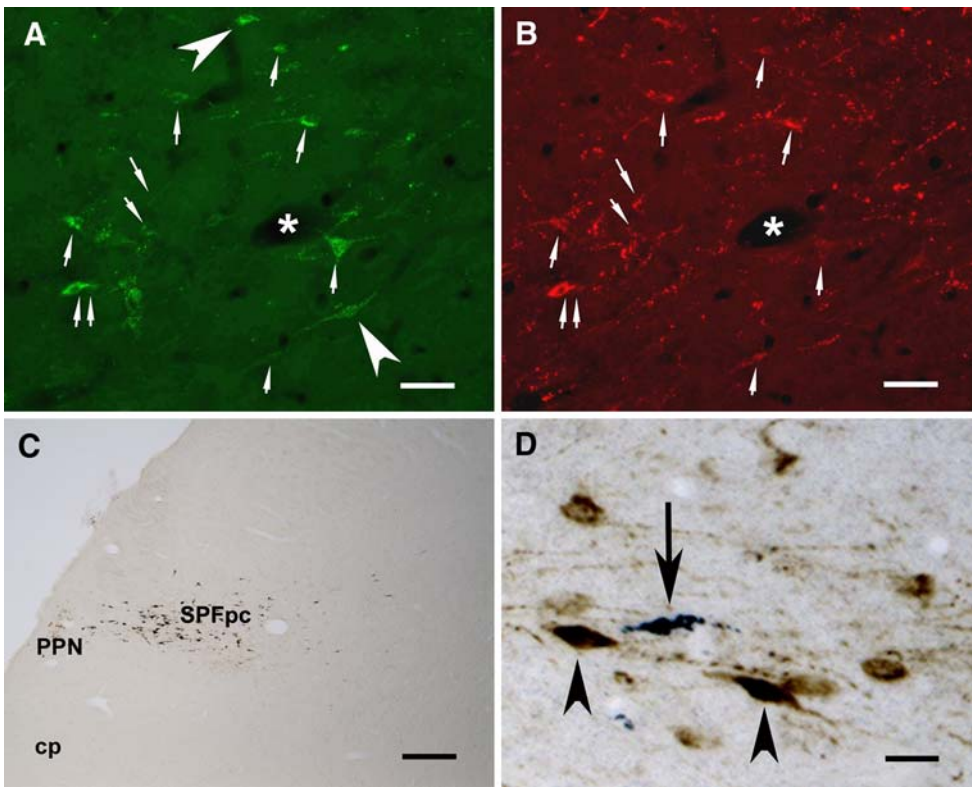

Fig. 4 Microphotographs showing double labeling in SPFpc nucleus. FG injected in ARC labeled cells in the mesencephalic SPFpc (**a**, **c**, and **d**). **a** Green fluorescence indicates FG containing cells. **b** *Red fluorescence* indicate TIP39 immunoreactivity in the same slide as (**a**). Major part of the FG labeled cells also contain TIP39. *Arrows* in the same position in (**a**) and (**b**) label the same cells. *Arrowheads* in (**a**) show cells, which contain FG but not TIP immunoreactivity. *Asterisk* (*) indicates the same vessel in (**a**) and (**b**). **c** Retrograde FG labeling (*bluish black color*) in SPFpc and CGRP immunoreactivity (*brown color*) in SPFpc and PPN. **d** A high power detail of SPFpc. *Arrow* shows FG labeled cell, *arrowheads* show cells containing FG and showing CGRP immunoreactivity as well (*dark blackish-brown color*). Abbreviation: *SPFpc* parvocellular part of subparafascicular nucleus. *Scale*: 75 μm in (**a**) and (**b**), 750 μm in **c** and 25 μm in (**d**)

associated with mating behavior, specifically with ejaculation in male rats and vaginocervical stimulation in females [26]. The same research group that carried out the previous experiment has also 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 [14]. Based on these observations, it seems that the lateral portion of the SPFpc blends into the PPN.

TIP39 is a recently characterized ligand of the parathyroid hormone 2 receptor. Dobolyi and his co-workers [13] have mapped the expression of this peptide in the rat brain and they found that a major population of TIP39 neurons resides in the SPF. Double immunostaining also showed that many TIP39 cells in the SPFpc are CGRP positive as well [13]. Our retrograde FG injections did label TIP-39 positive neurons in the SPFpc, just over the medial lemniscus.

Besides the discussed structures and factors many others may be involved in the PRL release. One of them is neuropeptide Y (NPY). Most of NPY containing neurons in the hypothalamus are located in the ARC. The hypothalamic level of NPY mRNA increases during lactation [27, 28]. In situ hybridization revealed that the elevation in the mRNA level mainly happened in the dorsomedial nucleus and in the dorso- and ventrolateral portion of the caudal half of ARC [29]. This latter is the same area to where FG was iontophoretically applied in our experiment. The increased level of NPY during lactation induces an increased food intake that is necessary for successful lactation [30]. CGRP induces hypoprolactinemia [31].

Tachykinins have been also demonstrated to stimulate OT and vasopressin in the paraventricular nucleus (PV), which in turn result in PRL release. Tachykinins also potentiate the stimulating effect of glutamate and VIP on PRL release [32]. Contradictory data also appeared in the literature demonstrating enhanced dopamine release from hypothalamic segments by tachykinins [33]. One of the tachykinins, neurokinin-B (NKB) immunoreactivity colocalizes with Dyn immunoreactivity in the ARC [34].

In summary, we proposed that besides the PPN, another nucleus, the SPFpc might also be involved in the pathway mediating suckling stimuli to the ARC. We could not confirm a direct connection between PPN and the ARC. In this study, we provided a morphological evidence that the adjacent SPFpc projects directly to the ARC. On the basis of previous and our recent studies, we propose that the pathway of suckling-induced PRL release may consist of six neurons (Fig. 5): (1) spinal ganglion, (2) posterior horn of the spinal cord, (3) lateral cervical nucleus, (4) PPN and SPFpc, (5) VMN and ventrolateral ARC, and finally (6) the TIDA neurons. The pathway crosses the midline between the spinal cord and the midbrain. The connections between the lateral cervical nucleus and the SPFpc, and between the VMN and the ARC on one hand and the lateral ARC and TIDA neurons on the other hand are not completely clarified at this time However, the above-mentioned physiological data strongly support our assumption.

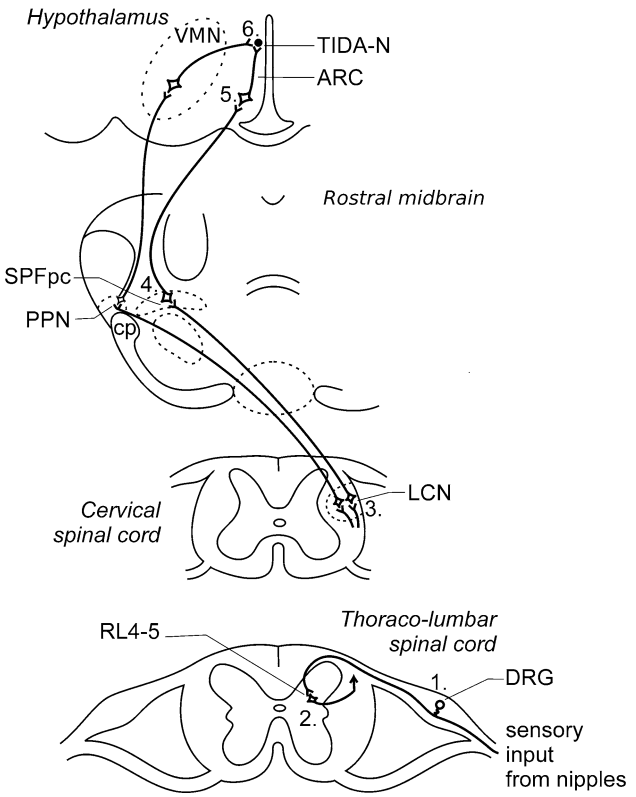

Fig. 5 Schematic illustration of our recent hypothesis on the ascending pathway from the nipples to TIDA neurons. First-order neurons (1) are in the dorsal root ganglia, second-order neurons (2) are in the dorsal horn, third orders (3) are in the lateral cervical nucleus of the spinal cord, fourth-order neurons (4) are located in the PPN and the SPFpc. These neurons project to the VMN and the ventrolateral part of Dyn immunoreactive arcuate neurons (fifth-order neurons, 5). Finally, Dyn neurons innervate TIDA neurons (sixth-order neurons, 6). A connection between the VMN and TIDA neurons is also proposed. Abbreviations: *ARC* arcuate nucleus; *cp* cerebral peduncle; *DRG* dorsal root ganglion; *LCN* lateral cervical nucleus; *PPN* peripeduncular nucleus; *RL4-5* Rexed laminae; *SPFpc* parvocellular part of subparafascicular nucleus; *TIDA-N* dopaminergic neuron; *VMN* ventromedial nucleus

Materials and methods

Animals

Adult non-lactating female Sprague-Dawley rats (3–4 months old) purchased from Zivic-Miller Laboratories, Inc. (Zelienople, PA) were housed on a 12 h light/12 h dark schedule (lights on from 3 am to 3 pm) and given free access to food and water. Temperature was maintained at 22 ± 2°C. The University of Maryland's committee on Animal Care and Use approved experimental paradigms according to NIH guidelines. After completing the presented study, the authors moved to other workplaces.

Stereotaxic interventions

Experiment 1

To determine the projections of neurons of the PPN and neighboring regions to the ARC, the primarily anterograde tracer BDA (Mol. Wt. 10,000, Sigma-Aldrich, St. Louis, MO) was administered into the ventrolateral tegmentum of the midbrain just caudal to the posterior intralaminar thalamic nuclei using iontophoresis and labeled fibers were looked for in the medial basal hypothalamus.

Experiment 2

The 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 PPN and the neighboring region.

Only those animals in which the injection site was located in the targeted areas were included in the experiments (seven animals in Experiment 1 and five animals in Experiment 2).

On the day of the surgery, animals were anesthetized using 4% chlorohydrate (1 ml/100 g) 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 coordinates: PPN—A 4.20 mm, L 3.50 mm, V 3.2, or ARC—A 6.20 mm, L 0.15 mm, V 0.25 mm to the interaural line, the midline and the base of the brain, respectively, according to The Stereotaxic Atlas by Paxinos and Watson [15]. The superior sagittal sinus was removed to expose the interhemispheric fissure, which was used as the reference midline. A glass micropipette (30 μm tip diameter) filled with 10% BDA or 2% FG dissolved in 0.9% saline was used to apply the tracers using positive pulses of 2 μA, alternating 5 s on and 5 s off for a total of 5 min. It was then removed and the scalp was closed with wound clips.

Perfusion and tissue sectioning

Ten to 14 days following surgery, the animals that received tracers were anesthetized with an overdose of sodium pentobarbital (100 mg/kg), the blood was flushed out by physiological saline containing 2% sodium nitrite solution and perfused transcardially with 0.1 M potassium-phosphate buffered saline (KPBS) containing 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) solution and 2.5% acrolein (Polysciences, Warrington, PA, USA) (pH 6.8). An additional rinse of physiological saline was used to remove residual PFA and acrolein [35]. Brains were immersed into a 30% sucrose solution and later the brains were cut in coronal plain on a freezing sliding microtome at 25 μm and collected in an ethylene-glycol (Sigma, St. Louis, MO, USA) containing cryoprotectant/anti-freeze solution [36]. Sections were stored at −20°C until use.

Immunohistochemistry

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 in KPBS for 20 min 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 h 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 0.175 M sodium acetate solution. BDA was visualized using nickel sulfate-3, 3-diaminobenzidine chromogen (DAB) (Sigma) with H_2O_2 in 0.175 M 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 extremely 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 concentration of 1:100,000 for 48 h at 4°C, then in goat anti-rabbit biotinylated secondary antibody solution (Vector Laboratories, Burlingame, CA) at a dilution 1:600. FG was visualized using ABC solution 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 extremely enhanced.

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, selected sections were rinsed and incubated with monoclonal TH antibody (Chemicon, Temecula, CA) at a dilution of 1:500,000 for 24 h. 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 h 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 h, then with goat anti-rabbit biotinylated serum. 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.

Experiment 2 To chemically characterize the FG labeled neurons retrogradely labeled from the ARC, we stained the sections for TIP-39, CGRP, or Gal immunoreactivity using double labeling immunohistochemistry.

A. We conducted FG/CGRP double staining of selected sections to find out whether FG and CGRP immunoreactivities colocalize in the targeted areas. The sections were first stained for FG as described above. For visualization of FG, we have used nickel intensified DAB chromogen. The sections were then incubated in rabbit CGRP antiserum (Chemicon, Temecula, CA) at a dilution of 1:30,000 for 24 h, then in goat biotinylated antibody at a dilution 1:600 for an hour. The final reaction product was visualized by ABC complex and DAB chromogen only.

B. FG/TIP39 double staining. The visualization of FG and TIP39 was carried out by double fluorescence staining. The sections were first incubated in FG antiserum as described above, 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. [37] at a dilution of 1:5,000. 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 h.

C. FG/Gal double labeling. The sections were first 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 the ABC technique, and DAB chromogen was applied without nickel intensification.

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