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Paralemniscal TIP39 is induced in rat dams and may participate in maternal functions

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Abstract

The paralemniscal area, situated between the pontine reticular formation and the lateral lemniscus in the pontomesencephalic tegmentum contains some tuberoin-fundibular peptide of 39 residues (TIP39)-expressing neurons. In the present study, we measured a 4 times increase in the level of TIP39 mRNA in the paralemniscal area of lactating mothers as opposed to nulliparous females and mothers deprived of pups using real-time RT-PCR. In situ hybridization histochemistry and immunolabeling demonstrated that the induction of TIP39 in mothers takes place within the medial paralemniscal nucleus, a cytoarchitectonically distinct part of the paralemniscal area, and that the increase in TIP39 mRNA levels translates into elevated peptide levels in dams. The paralemniscal area has been implicated in maternal control as well as in pain perception. To establish the function of induced TIP39, we investigated the activation of TIP39 neurons in response to pup exposure as maternal, and formalin injection as noxious stimulus. Both stimuli elicited *c-fos* expression in the paralemniscal area. Subsequent double labeling demonstrated that 95% of neurons expressing Fos in response to pup exposure also contained TIP39 immunoreactivity and 91% of TIP39 neurons showed *c-fos* activation by pup exposure. In contrast, formalin-induced Fos does not co-localize with TIP39. Instead, most formalin-activated neurons are situated medial to the TIP39 cell group. Our data indicate that paralemniscal neurons may be involved in the processing of maternal and nociceptive information. However, two different groups of paralemniscal neurons participate in the two functions. In particular, TIP39 neurons may participate in the control of maternal functions.

Keywords

Tuberoinfundibular peptide of 39 residues; Parathyroid hormone 2 receptor; Medial paralemniscal nucleus; Formalin pain stress; A7 noradrenergic cell group; Pontine tegmentum; Suckling

Introduction

The paralemniscal area is situated in the lateral ponto-mesencephalic tegmentum medial to the nuclei of the lateral lemniscus bordered caudally by the Kölliker-Fuse nucleus and the A7 noradrenergic cell group, medially by the oral part of the pontine reticular formation and the pedunculopontine tegmental nucleus, ventrally by the rubrospinal tract, and rostrally by the retrorubral field (Andrejik and Beitz 1985; Paxinos and Watson 2005). The functions of paralemniscal neurons have been examined for a long time (Fuse 1926; Papez 1926; Henkel and Edwards 1978; Herbert et al. 1997; Hage and Jurgens 2006; Hannig and Jurgens 2006). The paralemniscal area has been implicated in brainstem pain regulatory systems based on changes in the activity of its neurons in response to noxious stimuli (Hardy et al. 1983) and analgesic effects of its stimulation (Zhao and Duggan 1988; Haws et al. 1989). Neurons in the paralemniscal area have also been suggested to be involved in the control of maternal adaptations as Fos expression was found in this area in mother rats following pup exposure (Li et al. 1999b). We recently identified a cytoarchitectonically distinct part of the paralemniscal area, the medial paralemniscal nucleus (MPL) (Varga et al. 2008), which contained neurons expressing tuberoinfundibular peptide of 39 residues (TIP39). TIP39 was identified as an endogenous agonist of the parathyroid hormone 2 receptor (Usdin et al. 1999; Usdin 2000) and suggested to be involved in nociception (Dobolyi et al. 2002; LaBuda and Usdin 2004; Dimitrov et al. 2010), endocrine (Ward et al. 2001; Sugimura et al.

2003; Dimitrov and Usdin 2010), and auditory (Palkovits et al. 2004, 2009; Varga et al. 2008) regulations. Apart from the medial paralemniscal nucleus, TIP39 is expressed in only two brain regions, both located in the posterior thalamus (Dobolyi et al. 2003b). In the posterior intralaminar complex of the thalamus, which is situated ventromedial to the medial geniculate body, TIP39 levels were increased in lactating rat dams (Cservenak et al. 2010). In contrast, these changes were not present in the other posterior thalamic TIP39 cell group in the periventricular gray of the thalamus (Cservenak et al. 2010). As far as the function of medial paralemniscal TIP39 neurons, very limited information is available. We found that these neurons express *c-fos* in response to high-intensity auditory stimulus (Palkovits et al. 2004, 2009). However, we do not know if these neurons participate in the nociceptive functions of the paralemniscal area and whether they are activated in mother rats (Dobolyi et al. 2010). Therefore, in the present study, we addressed the following objectives: (1) to measure the level of TIP39 mRNA in the paralemniscal area in lactating and non-lactating mother rats as well as in nulliparous female rats using quantitative real-time RT-PCR, (2) to describe the distribution of TIP39 neurons in the paralemniscal area of lactating mother rats by means of in situ hybridization histochemistry, (3) to determine TIP39 immunoreactivity in the paralemniscal area of rat dams as compared to that in lactating mother and nulliparous female rats, (4) to identify the neurochemical character of neurons in the paralemniscal area of mother rats that demonstrate *c-fos* activation in response to pup exposure using double immunolabeling, (5) to identify neurons that are activated in response to formalin-induced pain in the paralemniscal area.

Materials and methods

Animals

All animal experimentation was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Experiments were carried out according to experimental protocols approved by the Animal Examination Ethical Council of the Animal Protection Advisory Board at the Semmelweis University, Budapest, and meet the guidelines of the Animal Hygiene and Food Control Department, Ministry of Agriculture, Hungary. A total of 87 adult female and 8 male Wistar rats (260–340 g body weight; Charles Rivers Laboratories, Hungary) were used. All of the animals were 90–120 days old when killed. Three rats per cage were kept on the standard laboratory conditions with 12-h light, 12-h dark periods (lights on at 6.00 a.m.), at $22 \pm 1^\circ\text{C}$ and supplied with food and drinking water ad libitum. Pregnant and mother rats as well as other animals during the experimental period were housed individually. Three mother rats were excluded from the study because they delivered less than 6 pups or some of their pups died. The number of pups was adjusted to 10 within 2 days of delivery. Rats were anesthetized with an intramuscular injection of an anesthetic mix containing 0.2 ml/300 g body weight ketamine (100 mg/ml) and 0.2 ml/300 g body weight xylazine (20 mg/ml) for surgery, perfusions and dissections, which took place at 9–10 days postpartum for mothers.

Microdissection of brain tissue samples

In one experiment, brains of eight primiparous lactating mothers, eight primiparous mothers separated from their pups immediately after parturition were removed on post-partum day 8–9 together with brains of eight age-matched nulliparous control female rats. In another experiment, brains of six formalin-injected and six control rats were used for microdissection. Coronal cuts were made from fresh brains at the rostral level of the pontine nuclei and 3 mm caudal to this level to include the paralemniscal area. A circular micropunch needle of 2 mm diameter was used subsequently to dissect the brain tissue containing the paralemniscal area. The left and right paralemniscal areas were pooled in the

first experiment but were separately processed following unilateral formalin injections. The dissected tissue samples were frozen, and stored at -80°C .

Real-time RT-PCR

Total RNA was isolated from the microdissected tissue containing the MPL using Trizol[®] Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After diluting RNA to $2\text{ }\mu\text{g}/\mu\text{l}$, it was treated with Amplification Grade DNase I (Invitrogen) and cDNA was synthesized with a Superscript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. After tenfold dilution, $2.5\text{ }\mu\text{l}$ of the resulting cDNA was used as template in multiplex PCR reactions using dual-fluorescence labeled TAQMAN probes for TIP 39 (6-FAM-CGCTAGCTGACGACGCGGCCT-TAMRA), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; JOE-ATGGCCTTCCGTGTTCCCTACCCCC-TAMRA) as described previously (Dobolyi et al. 2006). The primers for TIP39 (CTGCCTCAGGTGTTGCCCT and TGTAAGA GTCCAGCCAGCGG) were used at 300 nM whereas the primers for GAPDH (CTGAACGGGAAGCTCACTGG and CGGCATGTCAGATCCACAAC) were used at 150 nM concentration. PCR reactions were performed with iTaq DNA polymerase (Bio-Rad Laboratories, Hercules, CA, USA). Cycle threshold values (C_T values) were obtained from the linear region of baseline-adjusted amplification curves. Standard curves for TIP39 and GAPDH were used to calculate the amount of cDNA in the samples.

Pup exposure of mother rats for Fos activation study

Rat dams ($n = 8$) were deprived of pups on postpartum day 8–9 at 13:00. The pups were returned to four mothers the following day at 9:00, 20 h after being removed, while four control mother rats remained isolated. All four mothers accepted the pups and suckling started within 10 min. The eight animals were killed 22 h after removing the pups, perfused transcardially, and processed for Fos and TIP39 immunocytochemistry.

Formalin injection

Mother rats ($n = 26$), age-matched freely cycling nulliparous female rats ($n = 6$), and adult male rats ($n = 8$) participated in the formalin injection experiments. Buffered 0.4 ml formalin (4%) was injected intramuscularly into the left hind leg of 13 mother, 3 nulliparous female, and 4 male rats. The needle was inserted into the same position on other 13, 3, and 4 rats (as controls) without injecting any solution. A group of animals (4 formalin-injected and 4 control mothers, all nulliparous females and males) were perfused transcardially 2 h after the injections processed for Fos and TIP39 immunohistochemistry. Another group, nine formalin-injected and nine control mothers were allowed to survive for 3 days after formalin injection during the period which they were deprived of their pups. Microdissection of the left and right paralemniscal areas was performed on 6–6 animals for the measurement of TIP39 mRNA levels. The other 3–3 animals were perfused transcardially and processed for Fos and TIP39 immunohistochemistry.

Tissue collection for histology and immunohistochemistry

Rats were deeply anesthetized and perfused transcardially with 150 ml saline followed by 300 ml of ice-cold 4% paraformaldehyde in phosphate buffer (PB, pH 7.4). Brains were removed and postfixed in the same fixative solution for 24 h and transferred to PB containing 20% sucrose for 2 days. For side orientation on the histological sections in formalin-injected animals, a small sagittal incision was made throughout the tissue blocks ipsilateral to the injection sites. Serial coronal sections were cut at $50\text{ }\mu\text{m}$ on a sliding microtome (SM 2000R, Leica Microsystems, Nussloch, Germany) between 1.0 and 10.0

mm caudal to the bregma level. For immunohistochemistry, sections were collected in PB containing 0.1% sodium azide and stored at 4°C until further processing. For cresyl-violet and Luxol fast blue stainings, serial coronal sections were cut from 5-mm thick blocks derived from 4 brains while serial horizontal sections were cut from 5-mm thick blocks derived from 2 brains containing the medial paralemniscal nucleus. Each section from the six blocks was mounted consecutively on gelatin-coated slides, and dried.

Cresyl-violet and Luxol fast blue–cresyl-violet staining

Coronal and horizontal sections from 2-2 brains of lactating mothers were stained in 0.1% cresyl-violet dissolved in PB, then differentiated in 96% ethanol containing acetic acid. Sections were then dehydrated and coverslipped with Cytoseal 60 (Stephens Scientific, Riverdale, NJ, USA).

Myelinated fibers in coronal sections from two brains of lactating mothers were visualized with the sulphonated copper othalocyanine Luxol Fast Blue with a modification of the Kluver–Barrera method as described before (McIlmoyl 1965). Briefly, sections were stained in 0.1% Luxol fast blue dissolved in 96% ethanol containing 0.05% acetic acid, and differentiated in 0.05% lithium-chloride followed by 70% ethanol. Subsequently, sections were stained in 0.1% cresyl-violet as described above. At the end, sections were dehydrated and coverslipped as described above.

Immunohistochemistry

Immunolabeling of TIP39—Every fourth free-floating brain section of three primiparous lactating mothers, three primiparous mothers separated from pups immediately after parturition, and three age-matched freely cycling nulliparous control female rats were killed at 8–9 postpartum days and immunostained for TIP39, as described previously (Dobolyi et al. 2002, 2003b). TIP39 was detected with an affinity-purified antiserum from a rabbit immunized with rat TIP39 which can be absorbed with synthetic TIP39 (Dobolyi et al. 2002, 2003b) and labels cell bodies with the same distribution as observed by in situ hybridization histochemistry (Dobolyi et al. 2003b, 2006). This antiserum (1:3,000) was applied for 48 h at room temperature followed by incubation of the sections in biotinylated anti-rabbit secondary antibody (1:800 dilution; Vector Laboratories, Burlingame, CA, USA) and then in avidin–biotin–peroxidase complex (1:300; Vector Laboratories, Burlingame, CA, USA) for 2 h. Subsequently, sections were treated with fluorescein isothiocyanate (FITC)-tyramide (1:8,000) and H₂O₂ in Tris–hydrochloride buffer (0.1 M, pH 8.0) for 6 min, mounted on positively charged slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA, USA) and coverslipped in antifade medium (Prolong Antifade Kit, Molecular Probes, Eugene, OR, USA).

Fos immunohistochemistry—Every fourth free-floating section of four brains per group (mothers on postpartum day 8–9 deprived of pups for 22 h and mothers deprived of pups for 20 h followed by pup exposure) as well as 4 formalin-injected mothers, 3 formalin-injected non-lactating nulliparous females, and 4 formalin-injected males and their 11 controls were immunolabeled as described for TIP39 immunostaining except that a rabbit anti-Fos primary antiserum (1:25,000; sc-52; Santa Cruz Biotechnology, Delaware, CA, USA) was used and the labeling was visualized by incubation in 0.02% 3,3'-diaminobenzidine (DAB; Sigma), 0.08% nickel (II) sulfate and 0.001% hydrogen peroxide in PB, for 5 min. Sections were mounted, dehydrated and coverslipped with Cytoseal 60 (Stephens Scientific, Riverdale, NJ, USA).

Double immunolabeling of TIP39 and Fos—Every fourth free-floating sections of the 24 rats used for single labeling Fos were immunolabeled at first for TIP39, as described

above. Then, sections were placed in rabbit anti-Fos primary antiserum (1:10,000) for 48 h at room temperature. The sections were then incubated in Alexa 594 donkey anti-rabbit secondary antibody (1:500; Molecular Probes) for 2 h, washed in PB overnight, mounted and coverslipped.

Analysis of TIP39 and Fos double immunolabeling

A section containing the MPL was randomly selected from each side of the 16 mother brains double labeled for TIP39 and Fos. The total number of TIP39-ir neurons with an identifiable cell nucleus as well as the number of double-labeled cells was counted using 20× objective of an Olympus BX60 light microscope equipped with fluorescent epi-illumination and a filter that allowed us to see both green and red colors. Subsequently, the number of single-labeled Fos-ir and TIP39-ir cells was also calculated in the area where TIP39 neurons were distributed.

In situ hybridization histochemistry for TIP39

Brains of three primiparous lactating mothers, three primiparous mothers separated from pups immediately after parturition, and three age-matched nulliparous control female rats were dissected and frozen on postpartum day 8–9. The estrous stage of the control females was not analyzed because estrogen level did not have a detectable effect on TIP39 mRNA expression level (Dobolyi et al. 2006). In situ hybridization was performed as described previously (Dobolyi et al. 2002, 2003b). Briefly, serial coronal sections (12 µm) were cut, mounted, dried, and stored at –80°C until use. Every tenth section was used for labeling TIP39 mRNA. In situ hybridization protocols are described in detail on the website <http://intramural.nimh.nih.gov/lcmr/snge/Protocols/ISHH/ISHH.html>. [³⁵S] UTP-labeled riboprobes were generated using a MAXIscript transcription kit (Ambion, Austin, TX, USA) from TIP39 cDNA subcloned into TOPO TA vectors (Invitrogen) and hybridized at 1 million DPM activity per slide. A region of the rat TIP39 cDNA sequence corresponding to amino acids –55 to 37, where amino acid 1 is the first residue of mature TIP39, was used to generate probes. Slides were dipped in NTB nuclear track emulsion (Eastman Kodak), stored for 3 weeks at 4°C for autoradiography, developed and fixed with Kodak Dektol developer and Kodak fixer, respectively, counterstained with Giemsa and coverslipped. Typically three sections per brain contained TIP39 mRNA. A picture of the section containing the densest autoradiography signal for TIP39 within the MPL was taken for each animal.

Microscopy, photography and image processing

Sections were examined using an Olympus BX60 light microscope also equipped with fluorescent epi-illumination. Images were captured at 2,048 × 2,048 pixel resolution with a SPOT Xplorer digital CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA) using 4–40× objectives. Confocal images were acquired with a Nikon Eclipse E800 confocal microscope equipped with a BioRad Radiance 2100 Laser Scanning System using 60× objectives at an optical thickness of 2 µm. Contrast and sharpness of the images were adjusted using the “levels” and “sharpness” commands in Adobe Photoshop CS 8.0. Full resolution was maintained until the photomicrographs were cropped and assembled for printing, at which point images were adjusted to a resolution of 300 dpi. Drawings were prepared by aligning the pictures with corresponding schematics adapted from a rat brain atlas (Paxinos and Watson 2005).

Statistical analysis

Statistical analyses were performed using Prism 5 for Windows (GraphPad Software, Inc., La Jolla, CA, USA) for RT-PCR measurements. mRNA levels in the paralemniscal area of

the three groups (control female, lactating mother, and mothers deprived of pups) were compared using one-way ANOVA followed by Bonferroni post-tests for post hoc comparisons. mRNA levels of the four groups in the formalin-injected experiment (ipsilateral and contralateral paralemniscal areas of formalin-injected rats, ipsilateral and contralateral paralemniscal area of control rats) were compared using two-way ANOVA.

Results

The topography of medial paralemniscal TIP39 neurons in mother rats

Paralemniscal TIP39 neurons were distributed in the MPL located in the caudal part of the paralemniscal area. Cells of the MPL were distinguished from those in adjacent areas by their organization into dorsolaterally oriented cell columns in mothers (Fig. 1), like in male rats, as shown previously (Varga et al. 2008). The cell columns were separated by 20–50 μ m wide cell free zones. We observed no difference between the cyto- and myeloarchitectonics of the MPL in mothers as compared to males described previously (Varga et al. 2008). The MPL narrows dorsally between the caudal part of the pedunculopontine tegmental nucleus and the intermediate nucleus of the lateral lemniscus resulting in a cone shape of the nucleus in coronal sections (Fig. 1). The caudal border of the MPL is the A7 cell group medially and the Kölliker-Fuse nucleus laterally. Ventrally, the MPL borders on the rubrospinal tract (Fig. 1). These adjacent structures can be clearly distinguished by the abrupt end of the dorsolaterally oriented cell columns.

RT-PCR demonstrates an elevated level of TIP39 mRNA in mother rats

In the MPL, lactating mother rats had a 4.0 times higher level of TIP39 mRNA than age-matched nulliparous control female rats. In contrast, TIP39 mRNA level was as low as that in control females when the pups were taken away from mothers immediately after delivery (Fig. 2). The mRNA level of TIP39 (expressed as $100000 \times \text{mRNA level of TIP39/mRNA level of GAPDH}$) was 65 ± 23 (mean $\pm$ SE) in control female rats, 259 ± 46 in lactating mother rats and 62 ± 11 for mothers deprived of pups representing a significant increase in the mother rats with pups. The change was in TIP39 mRNA levels as there was no difference in the level of GAPDH mRNA between the three investigated groups.

The distribution of TIP39 mRNA expressing and TIP39-immunoreactive neurons in the paralemniscal area of mother rats

TIP39 mRNA-containing (Fig. 3a–c) were evenly distributed within the MPL. Apart from the MPL and the posterior thalamus, we detected no signal for TIP39 mRNA in the examined parts (1–10 mm caudal to the bregma level) of the brain in lactating mothers. This location of TIP39 mRNA-expressing neurons was the same as that described earlier in young adult male and female rats (Dobolyi et al. 2003b, 2006). However, the intensity of the autoradiography signal was markedly higher in the MPL of mother rats as compared to nulliparous females and pup-deprived mothers (Fig. 3a–c).

TIP39 immunoreactivity in the paralemniscal area in mother rats

The distribution of TIP39-immunoreactive neurons (Fig. 3d–f) within the paralemniscal area was similar to that of TIP39 mRNA-containing (Fig. 3a–c) neurons. The intensity of immunolabeling was increased in lactating mother rats as compared to control females and pup-deprived mothers (Fig. 3e). Thus, a large number (more than 30 per section) of TIP39-ir cell bodies were observed in the MPL of rat dams (Fig. 3e) while only a few (less than 10 per section) TIP39-ir cell bodies were detected in control female rats (Fig. 3d) and mothers separated from their pups immediately after delivery (Fig. 3f).

Fos activation in rat dams in response to pup exposure

Suckling started within 10 min of returning the pups to mothers for each dam. Intensely labeled Fos-ir neurons appeared in response to pup exposure in a number of brain regions including for example the medial preoptic nucleus, the anterior periventricular hypothalamic nucleus, the periaqueductal gray, and the lateral septal nucleus (not shown). Similar to these brain regions, very few Fos-ir neurons (less than 3 per section) were detected in the MPL of rat dams 22 h after separating them from their pups (Fig. 4). However, when the dams were exposed to their pups for 2 h following 20 h of separation, Fos-ir nuclei (over 30 per section) appeared in the MPL (Fig. 3). We observed that Fos-ir nuclei were evenly distributed within the MPL while other parts of the paralemniscal area remained devoid of Fos-positive cells, except for a few cells dorsal to the MPL. The number of TIP39-ir neurons with an identifiable nucleus in the MPL was 31 ± 3 (mean $\pm$ SE) in randomly selected sections (1 section from each side of 4 lactating mother) containing the MPL. Double labeling revealed that 91% of TIP39-ir neurons in the MPL were Fos-positive. The few TIP39-ir neurons whose cell bodies lacked Fos immunoreactivity did not seem to form a separate cell group but rather were present in all parts of the MPL. The co-localization between TIP39- and Fos-positive neurons was very high as 95% of neurons expressing Fos in response to pup exposure-contained TIP39 in pup-exposed mothers (Fig. 4).

Fos activation in response to formalin injection

Formalin injection into the left hind leg of rats resulted in Fos expression in several parts of the brain 2 h after the injections (not shown). A similar distribution was found for males, females, and mothers. In particular, the hypothalamic paraventricular nucleus exhibited a bilateral induction of Fos while a predominantly contralateral induction of Fos was found in some parts of the lateral parabrachial nucleus and the locus coeruleus. In addition, we also observed an intensely labeled Fos-positive cell group immediately medial to the MPL (Fig. 5) in formalin-injected animals. These Fos-positive cells appeared almost exclusively contralateral to the injection site. Double labeling of Fos and TIP39 revealed that neither co-localization nor even a partial overlap exists between Fos and TIP39-positive cells (Fig. 5c, d). FA lower density of Fos-positive neurons was also apparent dorsal to the MPL. In control rats, the induction of Fos immunoreactivity was not observed in the hypothalamic paraventricular nucleus, the lateral parabrachial nucleus, the locus coeruleus, and the area medial to the MPL. However, lightly labeled Fospositive neurons were apparent dorsal to the MPL.

Three days following the formalin injection, Fos-positive neurons were not present in the MPL and medial to the MPL (Fig. 6a, b). However, in the area dorsal to the MPL, some Fos-positive neurons were observed. Their distribution was similar in formalin-injected and control rats (Fig. 6a, b).

TIP39 levels in the paralemniscal area 3 days following formalin injection

A small number of TIP39-ir cell bodies (less than 10 per section) were detected in the MPL on either side of the brain 3 days following formalin injections (Fig. 6c). The number of TIP39-ir cell bodies was similarly low in control rats who did not receive formalin (Fig. 6d). Apart from the few cell bodies, TIP39 fibers exhibiting a relatively low-intensity immunolabeling were also present in the MPL. Their distribution pattern and labeling intensity appeared similar in formalin-injected and control animals (Fig. 6c, d).

In the MPL, TIP39 mRNA levels did not differ between formalin-injected and control groups 3 days after injections and were similarly low as in nulliparous females. The mRNA level of TIP39 (expressed as $100000 \times \text{mRNA level of TIP39/mRNA level of GAPDH}$) was

61 ± 18 (mean ± SE) in the ipsilateral and 78 ± 34 in the contra-lateral MPL of formalin-injected rats, 105 ± 41 in the ipsilateral and 71 ± 27 in the contralateral MPL of control rats.

Discussion

We first discuss the expressional changes of TIP39 in the MPL of mothers. Next, we interpret activational data in relation to the maternal and nociceptive functions of paralemniscal neurons. Finally, we describe the potential maternal functions of TIP39 neurons in the MPL.

Induction of TIP39 in the paralemniscal area of mother rats

The level of TIP39 mRNA was elevated specifically in the presence of pups while TIP39 mRNA levels were at their low, basal, non-maternal level in the absence of pups. Thus, the increase in the level of TIP39 mRNA is a temporary phenomenon during lactation. The induction is likely to take place in all TIP39 neurons within the MPL as suggested by the increased autoradiography signal in the observed TIP39-expressing neurons following in situ hybridization histochemistry. In turn, the distribution of TIP39 mRNA-expressing cells in the paralemniscal area suggests that no additional, TIP39-negative cells are recruited in mothers. The increased TIP39 immunoreactivity in rat dams suggests that the increase in TIP39 mRNA level translates into elevated peptide level, which in turn suggests a function of the induced TIP39 in mother rats. A function of the induced TIP39 is also conceivable because the expression level of the receptor of TIP39, parathyroid hormone 2 receptor does not decrease during postnatal development as TIP39 does (Dobolyi et al. 2006). Thus, parathyroid hormone 2 receptor is available for maternally induced TIP39 to exert its actions.

The temporal pattern of activation of paralemniscal TIP39 neurons was similar to that described previously for one of the posterior thalamic TIP39 cell groups (Cservenak et al. 2010). In the posterior intralaminar complex of the thalamus, a presumed relay station of the suckling reflex, TIP39 neurons also demonstrated an elevated TIP39 level during lactation. In contrast, TIP39 expression was not affected by lactation in the third group of TIP39 neurons in and around the magnocellular subparafascicular nucleus in the periventricular gray of the thalamus (Cservenak et al. 2010). The medial paralemniscal and posterior intralaminar thalamic TIP39 neurons belong to different groups of cells because the two nuclei are situated over 3 mm from each other and demonstrate markedly different cytoarchitectonics (Dobolyi et al. 2010) and afferent neuronal connections (Coolen et al. 2003; Varga et al. 2008). Nevertheless, the similar induction pattern of TIP39 in these 2 nuclei suggests that they participate in related neuronal functions.

Activation of paralemniscal TIP39 neurons in mother rats

The appearance of Fos in response to pup exposure represents the activation of those neurons as Fos is the protein product of *c-fos*, a well-known immediate early gene that appears in activated neurons (Bullitt 1990; Morgan and Curran 1991; Herdegen and Leah 1998). Fos appeared in the paralemniscal area of mother rats when they were exposed to their litter. The location of activated neurons was similar to that reported previously for neurons activated by suckling (Li et al. 1999b). We identified this location as the MPL. Within the MPL, Fos was located in TIP39 neurons, which is the major neuronal cell group of this nucleus (Varga et al. 2008). Based on the very low number of Fos-positive but TIP39-immunonegative neurons, it is likely that other cell types within the MPL are generally not activated in mother rats.

Pup exposure represents a complex stimulus for the mothers. Theoretically, olfactory, somatosensory, visual and auditory inputs from the pups could all contribute to the activation of TIP39 neurons in the MPL by increasing their neuronal activity via a specific circuitry. Suckling is the most dominant stimulus for mothers, which contributes to a variety of central maternal adaptations (Russell et al. 2001). Indeed, suckling represents one of the stimuli that activates paralemniscal TIP39 neurons. Its importance is also emphasized by the previous data reporting that exteroceptive sensory stimuli associated with pup exposure without suckling did not evoke Fos expression in the MPL (Li et al. 1999b). However, auditory input could also contribute to the activation of TIP39 neurons in the MPL because they receive massive input from the auditory cortex, the inferior colliculus and the periolivary area (Varga et al. 2008), and can be activated by highly intense noise stimulus (Palkovits et al. 2009). Furthermore, an indirect activation of paralemniscal TIP39 neurons via maternal hormones cannot be excluded either. Nevertheless, the activation of paralemniscal TIP39 neurons in response maternal stimuli suggests that TIP39 is involved in the processing of maternal information.

Potential maternal functions of paralemniscal TIP39 neurons

Limited information is available regarding the functions of the MPL and TIP39 neurons located there. The position of the MPL immediately next to the nuclei of the lateral lemniscus and its bilateral anatomical connections with auditory brain regions (Dobolyi et al. 2003a; Varga et al. 2008) suggest some auditory functions of paralemniscal TIP39 neurons. In bats, the paralemniscal area medial to the intermediate nucleus of the lateral lemniscus is activated by ultrasounds and plays a role in vocalization (Metzner 1993; 1996; Fenzl and Schuller 2002). In rats, paralemniscal TIP39 neurons were specifically activated by high-intensity noise (Palkovits et al. 2009). The current results on the lack of activation of paralemniscal TIP39 neurons in a painful condition suggest that it is not the nociceptive aspect of the high-intensity noise that activated TIP39 neurons in the MPL. However, specific auditory inputs may play a role in the activation of TIP39 neurons in mothers. Rat pups, when isolated, are known to vocalize in the ultrasonic range (Hofer 1996). Pup ultrasonic vocalizations have been described to induce prolactin secretion and maternal behaviors in rats (Terkel et al. 1979; Hashimoto et al. 2001). Still, there are no data available at present on the anatomical pathway how ultrasonic vocalization reaches limbic and hypothalamic centers responsible for maternal behavioral and neuroendocrine changes. We hypothesize that paralemniscal TIP39 neurons may be activated in mother rats by ultrasonic vocalization of pups. In turn, paralemniscal TIP39 neurons could mediate pup ultrasonic vocalization towards higher brain centers of their mothers thereby contributing to central maternal adaptations.

Unfortunately, the projections of paralemniscal TIP39 neurons are not fully established yet. Stereotaxic lesion studies suggest projections to non-tonotopic auditory brainstem regions (Dobolyi et al. 2003a) while studies using retrograde tracers suggest that paralemniscal fibers may reach hypothalamic targets such as the arcuate nucleus (Li et al. 1999a) and the hypothalamic paraventricular nucleus (Palkovits et al. 2004). Projections of paralemniscal neurons to non-tonotopic auditory brainstem regions (Dobolyi et al. 2003a) might sensitize the maternal auditory system to pup vocalization. In turn, projections to the hypothalamic paraventricular nucleus (Palkovits et al. 2004) might be involved in the altered maternal stress response while projection of paralemniscal neurons to the arcuate nucleus (Li et al. 1999a) might contribute to prolactin release.

Pain-related paralemniscal activation

Formalin injection induces pain and also leads to a stress response of the animals (Capone and Aloisi 2004). In our experiments, the distribution of Fos immunoreactivity throughout a

number of brain areas was similar as described previously following formalin injection (Lanteri-Minet et al. 1994; Bester et al. 1997; Palkovits et al. 1997; Pacak et al. 1998). The appearance of Fos in the hypothalamic paraventricular nucleus suggested that our injections resulted in a stress response of the animals as expected. Brain regions participating in the transmission of nociceptive signals such as parts of the lateral parabrachial nucleus were also activated. In addition, we found activated neurons in the paralemniscal area contralateral to the side of formalin injection suggesting that they receive specific nociceptive information. A similar number and distribution of activated cells in males, females, and mothers suggest that the function of these cells is not related to gender and reproductive status. We also showed that TIP39 neurons in the MPL do not demonstrate *c-fos* activation following formalin stress and the mRNA level of TIP39 is not elevated by formalin injection suggesting that TIP39 neurons are not involved in pain-related stress responses. In turn, the distribution of formalin-activated paralemniscal neurons overlaps, at least partly with that of noradrenergic neurons belonging to the A7 cell group. In this area, tyrosine hydroxylase-positive noradrenergic as well as tyrosine hydroxylase-negative neurons were suggested to be activated by descending nociceptive input from the periaqueductal gray (Bajic and Proudfit 1999; Bajic et al. 2001; Bajic and Commons 2010). A7 noradrenergic neurons are known to project to the spinal cord and may participate in descending nociceptive control (Fritschy and Grzanna 1990; Pertovaara 2006; Min et al. 2008).

In conclusion, we have provided data indicating that TIP39 becomes available in paralemniscal neurons of mother rats. Furthermore, we showed that paralemniscal neurons are activated in response to maternal and nociceptive inputs as well suggesting that they may be involved in the corresponding functions. However, two different groups of paralemniscal neurons participate in the two functional activities. TIP39 neurons in the medial paralemniscal nucleus are activated in mother rats and are likely to be involved in the processing of some aspects of maternal adaptations.

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Fig. 1.

TIP39 neurons in the paralemniscal area of mother rats in a coronal plane. The drawing in **a** is adapted and modified from a rat brain stereotaxic atlas of Paxinos and Watson (2007). The *red circle* corresponds to the area dissected for RT-PCR. **b–d** Correspond to the *framed area* in the *right side* of **a**. The *dotted line* in these *panels* delineates the medial paralemniscal nucleus (MPL). **b** A photomicrograph of a section stained with cresyl-violet demonstrates the cytoarchitectonics of the MPL, in which distinct column-like structure is a characteristic feature. **c** A photomicrograph of a section stained with Luxol fast blue-cresyl violet demonstrates the myeloarchitectonics of the MPL. Dorsoventrally projecting massive bundles of the lateral lemniscus are evident lateral to the MPL. The rostrocaudally projecting fibers within the rubrospinal tract (rs) clearly indicates the ventral border of the MPL. **d** TIP39-immunolabeled neurons (*black*) are present in the MPL as shown in a section lightly counterstained with Luxol fast blue. *IC* inferior colliculus, *ILL* intermediate nucleus of the lateral lemniscus, *PnO* oral part of the pontine reticular formation, *scp* superior cerebellar peduncle. *Scale bar* 400 μ m for **b–d**

Fig. 2.

TIP39 is induced in the MPL of rat dams as demonstrated by quantitative real-time RT-PCR. **a** In the MPL, the level of TIP39 mRNA is significantly higher ($***p < 0.001$) in lactating mothers than in age-matched nulliparous control female rats and mother rats deprived of pups immediately after delivery ($n = 8$ in each group) as revealed by using one-way ANOVA ($F = 15.81$). Bonferroni post-tests for post hoc comparisons further demonstrated that TIP39 mRNA level in the lactating mother was significantly ($p < 0.001$) higher than that in control female rats ($t = 5.21$) and mothers deprived of their pups ($t = 5.04$) while the TIP39 mRNA levels did not differ in the latter two groups ($t = 0.09$). Data are expressed in the ratio of TIP39 to GAPDH mRNA observed in the hypothalamic paraventricular nucleus, the lateral parabrachial nucleus, the locus coeruleus, and the area medial to the MPL. However, lightly labeled Fos-positive neurons were apparent dorsal to the MPL.

Fig. 3.

The induction and distribution of TIP39 within the medial paralemniscal nucleus (MPL). An increased level of TIP39 mRNA expression is shown in the MPL of mother rats in bright-field photomicrographs of in situ hybridization histochemical sections (**a–c**) and in sections fluorescent immunolabeled for TIP39 (**d–f**). **a** TIP39 mRNA signal is barely detectable in the MPL of a 4-month-old control nulliparous female rat. **b** Intense autoradiography signal is shown for TIP39 in all parts of the MPL in lactating mother rats. **c** In mother rats whose pups had been removed immediately after delivery, TIP39 mRNA signal is as low as in the nulliparous control female. TIP39 immunoreactivity in the MPL changes in parallel with mRNA levels. **d** The MPL contains only a low density of weakly immunolabeled neuronal cell bodies the control female rats. **e** Intensely immunolabeled TIP39 cell bodies are distributed in all parts of the MPL in lactating mother. **f** After removal of the pups, the intensity of the immunolabeling was reduced to the level of the control female shown in **d**. *ILL* intermediate nucleus of the lateral lemniscus, *rs* rubrospinal tract. Scale bars 400 μ m for each panel

Fig. 4.

Fos expression is demonstrated in response to pup exposure in the medial paralemniscal nucleus (MPL) of mother rats. **a** Fos-ir neuronal cell bodies are present in the medial paralemniscal nucleus (MPL) of mother rats 2 h after returning their pups following their removal for 20 h. **b** Fos-immunopositive neurons are absent in the MPL of mother rats deprived of pups for 22 h. **c, d** Double fluorescent immunolabeled sections of mother rats demonstrate that most Fos-expressing cells contain TIP39 in the MPL. **c** The low magnification photomicrograph shows that the distribution of green TIP39-ir cell bodies and red Fos-ir nuclei overlaps. **d** The confocal image shows that the vast majority of TIP39-labeled cells contain red labeled Fos protein in their nuclei. *ILL* intermediate nucleus of the lateral lemniscus, *rs* rubrospinal tract. *Scale bars* 500 μm for **a–c**, and 100 μm for **d**

Fig. 5.

Fos expression is demonstrated in the paralemniscal area in response to formalin injection into the left hind leg of mother rats. **a** Fos-ir neuronal cell nuclei are present in the contralateral paralemniscal area following formalin injection. Most of the labeled cells are situated medial to the medial paralemniscal nucleus (MPL) while a lower number of Fos-ir cells are located dorsal to the MPL and a very low number of Fos-ir cells are located within the MPL. **b** In controls without formalin injection, only a few, lightly labeled Fos-ir neurons are present in the paralemniscal area. Most of these cells are situated dorsal to the MPL. **c** Double immunolabeling demonstrates that Fos-expressing cells are situated medial to the TIP39 neurons distributed within the MPL. **d** The confocal image shows that TIP39 neurons do not contain Fos immunoreactivity. *ILL* intermediate nucleus of the lateral lemniscus, *rs* rubrospinal tract. *Scale bars* 500 μ m for **a–c**, and 100 μ m for **d**

Fig. 6.

Fos and TIP39 immunoreactivities in the right paralemniscal area 3 days after formalin injection into the left hind leg of mother rats. **a** Fos-ir neuronal cell nuclei are absent in the medial paralemniscal nucleus (MPL) and the area medial to it 3 days after formalin injection. Some labeled cells are situated dorsal to the MPL. **b** In controls without formalin injection, the distribution of Fos-ir cell nuclei is the same as after formalin injection. Thus Fos-ir cell nuclei are restricted to the area dorsal to the MPL. **c** Relatively lightly labeled TIP39-ir fibers and few cell bodies are present in the MPL 3 days after formalin injection. **d** In controls without formalin injection, the distribution pattern and the intensity of TIP39 immunoreactivity in the paralemniscal area is similar to that of formalin-injected rats. *ILL* intermediate nucleus of the lateral lemniscus, *rs* rubrospinal tract. *Scale bar* 500 μ m