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POSTNATAL DEVELOPMENT AND GENDER DEPENDENT EXPRESSION OF TIP39 IN THE RAT BRAIN

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Abstract

Tuberoinfundibular peptide of 39 residues (TIP39) is a selective agonist of the parathyroid hormone 2 (PTH2) receptor. The topographical distributions of TIP39 and the PTH2 receptor in the brain, described in young male rats, suggested that TIP39 has limbic and endocrine functions. In the present study, we investigated the expression of TIP39 and the PTH2 receptor in male and female rat brain during postnatal development by means of *in situ* hybridization histochemistry, quantitative RT-PCR and immunocytochemistry. TIP39's distribution and expression levels are similar in young female and male brains. TIP39 mRNA levels peak at postnatal day 14 and subsequently decline both in the subparafascicular area and the medial paralemniscal nucleus, the two major sites where TIP39 is synthesized. A greater developmental decrease in TIP39 expression in males leads to greater levels in older females than older males. The decrease is partially reversed by pre-pubertal but not post-pubertal gonadectomy. TIP39 peptide levels in cell bodies change in parallel with mRNA levels, while TIP39 appears and disappears somewhat later in nerve fibers. In addition, TIP39 peptide levels are also sexually dimorphic in older rats. In contrast, PTH2 receptor expression in the brain does not decrease during puberty and is not sexually dimorphic even in old animals. The appearance of TIP39 during early, and decline during late, postnatal development together with the gender dependent levels in mature animals suggest that TIP39 may play a role in sexual maturation or gender specific functions.

Indexing terms

tuberoinfundibular peptide of 39 residues; parathyroid hormone 2 receptor; TIP39 and PTH2 receptor *in situ* hybridization and immunocytochemistry and quantitative real; time RT-PCR; transient expression; ontogeny; puberty; sexual dimorphism; gonadectomy

INTRODUCTION

Tuberoinfundibular peptide of 39 residues (TIP39) was purified from bovine hypothalamus on the basis of its activation of the parathyroid hormone 2 (PTH2) receptor (Usdin et al., 1999b). Mouse, rat, human and zebrafish sequences have been reported (Dobolyi et al., 2002; Hansen et al., 2002; John et al., 2002; Papasani et al., 2004). TIP39 has limited common sequence with parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) and a similar three-dimensional structure (Piserchio et al., 2000). These

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three members define a small peptide family (Usdin et al., 2000). PTH and PTHrP are endogenous ligands of the parathyroid hormone 1 (PTH1) receptor, while TIP39 is a selective agonist of the PTH2 receptor (Usdin, 2000) and is a strong candidate for its endogenous ligand (Usdin et al., 2003).

The expression of TIP39 in the central nervous system is restricted to two major sites, the subparafascicular area in the caudal thalamus and the medial paralemniscal nucleus in the lateral pons (Dobolyi et al., 2003b; Dobolyi et al., 2002). The distributions of TIP39 fibers (Dobolyi et al., 2003b) and the PTH2 receptor (Wang et al., 2000) in the central nervous system have recently been described in young male rats. PTH2 receptors and TIP39 fibers have similar distributions in many brain regions, suggesting that they form a neuromodulator system (Dobolyi et al., 2006). TIP39 fibers and PTH2 receptors are abundant in the medial prefrontal cortex, the lateral septum, the amygdala, several different hypothalamic nuclei, the paraventricular thalamic nucleus, and the parabrachial nuclei (Dobolyi et al., 2006; Dobolyi et al., 2003b; Wang et al., 2000) suggesting limbic-endocrine functions for TIP39 (Dobolyi et al., 2003a; Usdin et al., 2003). Initial functional studies suggest that TIP39 is involved in the transmission of nociceptive information towards higher nociceptive centers (Dobolyi et al., 2002; LaBuda and Usdin, 2004). In addition, TIP39 may modulate an affective component of nociception within the brain (LaBuda and Usdin, 2004). TIP39 may also be involved in the hypothalamic regulation of pituitary hormones (Sugimura et al., 2003; Usdin et al., 2003; Ward et al., 2001) and the audiogenic stress response (Palkovits et al., 2004). Centrally administered TIP39 increased the plasma level of adrenocorticotropin and luteinizing hormone (Ward et al., 2001), inhibited the release of arginine vasopressin (Sugimura et al., 2003) and growth hormone (Usdin et al., 2003), and produced anxiolytic- and antidepressant-like effects (LaBuda et al., 2004). Areas in which TIP39 neurons are concentrated contain cells that are specifically activated following ejaculation in male rats (Coolen et al., 2004) and in humans (Holstege et al., 2003), or during lactation (Lin et al., 1998). Since TIP39 cells are major output neurons of these regions (Dobolyi et al., 2003a; Wang et al., 2006b), TIP39 might be involved in mediating some aspects of these sexual and maternal behaviors. Many sexual, maternal and other limbic-endocrine functions undergo changes during postnatal development and demonstrate gender differences. However, no information is available on the developmental pattern of TIP39 and PTH2 receptor expression or on their expression in female brains. This missing data hinders further investigation of the role of TIP39 in the above listed sexual, maternal and limbic-endocrine functions. Therefore, in the present study, we addressed the following questions:

1. How does TIP39 mRNA expression change during postnatal development and the period of sexual maturation?
2. Is TIP39 mRNA expression different in male and female rat brain? Does it change in response to gonadectomy?
3. Do changes in TIP39 peptide levels follow changes in TIP39 mRNA expression?
4. Does PTH2 receptor expression change during the period of sexual maturation in male and female?

To address these questions we performed *in situ* hybridization histochemistry for TIP39 and the PTH2 receptor at different ages in male and female and also in gonadectomized rats. We confirmed and quantified our expression data by real-time RT-PCR. In addition, we examined TIP39 and PTH2 receptor immunoreactivity in cell bodies as well as in fibers using immunocytochemistry.

MATERIALS AND METHODS

Animals

All procedures were performed according to approved National Institutes of Mental Health (Bethesda, MD, USA) animal care protocols, and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed on Sprague-Dawley rats (Taconic, Germantown, NY). All efforts were made to minimize the number of animals used and their suffering. A total of 252 rats were divided into 11 age/gonadectomy groups based on euthanasia at postnatal day (PND) - 1, 7, 14, 33, 57, 125 or 300, or pre-pubertally or post-pubertally gonadectomized and gonadectomy control and euthanized at PND 300. Animals in each of the 11 groups were used for *in situ* hybridization (2 males and 2 females), RT-PCR (6 males and 6–12 females), and immunocytochemistry (3 males and 3 females). Rats were anaesthetized with sodium pentobarbital (80 mg/kg i.p.) and decapitated for *in situ* hybridization and RT-PCR or perfused for immunocytochemistry. The estrous cycle stage (diestrus, proestrus, estrus and metestrus) of PND-57 and PND-125 female rats was identified from vaginal smears obtained before sacrificing the animals.

Gonadectomy

Rats were anaesthetized by i.p. injection of 50 mg/kg sodium pentobarbital at PND-24 (prepubertal gonadectomy) and PND-55 (postpubertal gonadectomy). Female rats were ovariectomized through bilateral upper flank incisions (Wayneorth and Flecknell, 1992). The ovarian bundles were tied off and the ovaries removed. The fascia was closed with sutures and the skin closed with metal clips. Male rats were castrated through a single scrotal incision (Wayneorth and Flecknell, 1992). The testicular bundles were ligated before removing the testes, and the skin closed with sutures.

In situ hybridization histochemistry

Brains of 2 male and 2 female rats per group were removed and the fresh tissue quickly frozen on dry ice. Coronal sections (12 μ m) were cut using a cryostat from bregma level -3.5 mm to -10 mm, mounted on positively charged slides (SuperfrostPlus®, Fisher Scientific, Pittsburgh, PA), dried, and stored at -80°C until use. *In situ* hybridization protocols are described in detail on the World Wide Web (<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) from polymerase chain reaction-amplified fragments of the TIP39 cDNA subcloned into the vector pBluescript (Stratagene, La Jolla, CA). Antisense or sense (control) riboprobes were prepared using T7 or T3 RNA polymerase, respectively. 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. We have shown previously that this antisense probe produces equivalent hybridization patterns to probes with non-overlapping sequences corresponding to amino acids -55 to -18, and -17 to 37 (Dobolyi et al., 2003b). Similarly, a region of the rat PTH2 receptor cDNA sequence corresponding to bases 482–864 was used to generate probes. We have shown previously that this antisense probe produces equivalent hybridization patterns to probes with the non-overlapping sequence corresponding to bases 1274–1828 (Wang et al., 2000). Following hybridization and washes, slides were dipped in NTB2 nuclear track emulsion (Eastman Kodak) and stored at 4°C for 3 weeks. Then, the slides were developed and fixed with Kodak Dektol developer and Kodak fixer, respectively, and counterstained with Giemsa.

Real-time RT-PCR

The estrous cycle stage of PND-57 and PND-125 female rats were identified from vaginal smear on the day of sacrificing the animals. At least 2 animals at all 4 stages of the estrous cycle but no more than 4 animals with the same cycle stage were included in a group. Upper brainstem comprised of hypothalamus, thalamus and midbrain was dissected to include all TIP39 cells in the subparafascicular area. Cortex and hippocampus were removed, and the brainstem was dissected with coronal cuts immediately rostral to the optic decussation and immediately rostral to the pontine base. Pons was dissected to include all TIP39 cells in the medial paralemniscal nucleus. Cerebellum was removed, and the pons was dissected with coronal cuts immediately rostral to the pontine base and at the level of the facial nucleus and its root. Dissected tissue was kept at -80°C before total mRNA was isolated using Trizol^R Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After diluting mRNA to 2 $\mu\text{g}/\mu\text{l}$, RNA was treated with Amplification Grade DNase I (Invitrogen) and cDNA was synthesized with a ThermoscriptTM RT-PCR System (Invitrogen) according to the manufacturer's instructions. After 10-fold dilution, this cDNA was used as template in multiplex real-time PCR reactions performed with the DNA Engine Opticon^R 2 System (MJ Research, Reno, NV) using dual-fluorescence labeled probes for TIP39 (6-FAM-CGCTAGCTGACGACGCGGCCT-TAMRA), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; JOE-ATGGCCTTCCGTGTTCCCTACCCCC-TAMRA). The primers for TIP39 (CTGCCTCAGGTGTTGCCCT and TGTAAGAGTCCAGCCAGCGG) were used at 300 nM final concentration whereas the primers for GAPDH (CTGAACGGGAAGCTCACTGG and CGGCATGTCAGATCCACAAC) were used at 150 nM final concentration. The PCR was performed with Immomix (Bioline, Randolph, MA) under the following conditions: 95 $^{\circ}\text{C}$ for 7 min, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 0.5 min and 60 $^{\circ}\text{C}$ for 2 min. The PCR products were regularly run on gels to check for potential genomic DNA contamination, identified by its larger size.

Immunocytochemistry

TIP39 was detected with an affinity-purified antiserum from a rabbit immunized with rat (r) TIP39 coupled to keyhole limpet hemocyanin by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. This antiserum has previously been used and characterized (Dobolyi et al., 2003b; Dobolyi et al., 2002). The titer (50% maximum binding to immobilized peptide) of the affinity-purified anti-rTIP39 antiserum against rTIP39 was 3 ng/ml (Dobolyi et al., 2002). Immunolabeling with the affinity-purified anti-rTIP39 was abolished by pre-incubation with 1 μM synthetic rTIP39. The anti-rTIP39 antiserum exhibited less than 1% cross-reactivity with parathyroid hormone and no detectable cross-reactivity with other peptides tested including parathyroid hormone related peptide, calcitonin, substance P, vasoactive intestinal peptide, glucagon, and calcitonin gene-related peptide (Dobolyi et al., 2002). The anti-rTIP39 antiserum labels cell bodies with exactly the same distribution as observed by *in situ* hybridization histochemistry with probes directed against TIP39 mRNA, in the adult male (Dobolyi et al., 2003b), and also in adult females and young rats of both genders (present study).

Three rats per group were anaesthetized and perfused transcardially with heparinized saline (volume/ml: one third of body weight/g) followed by ice-cold buffered (pH=7.4) 4% paraformaldehyde (volume/ml: 1.3 x body weight/g). The brains were removed, postfixed in buffered 4% paraformaldehyde overnight, washed for at least 3 days with phosphate buffered saline (PBS, pH=7.4) and then 50- μm thick sections were cut with vibratome from bregma level -3.5 mm to -10 mm for coronal brain sections. Free-floating sections were then pretreated with 1% bovine serum albumin in PBS containing 0.6% Triton X-100 for 30 min at room temperature. The sections were then placed in anti-TIP39 primary antiserum (1:3000 for tyramide amplification and 1:600 for DAB reaction) for 48 hours at room

temperature or anti-PTH2 receptor primary antiserum (1:60000 for tyramide amplification and 1:15000 for DAB reaction) for 24 hours at room temperature as described previously (Dobolyi et al., 2006; Usdin et al., 1999a; Usdin et al., 1999b; Wang et al., 2000). The sections were then incubated in biotinylated anti-rabbit secondary antibody (1:600 dilution; Vector Laboratories, Burlingame, CA) for 2 hours followed by incubation in a solution containing avidin-biotin-peroxidase complex (1:150; Vector Laboratories, Burlingame, CA) for 2 hours. The sections were then treated with 0.06% DAB or FITC-tyramide (1:20000) and H₂O₂ in Tris hydrochloride buffer (0.1 M, pH 8.0) for 10 minutes as described previously (Hunyady et al., 1996), mounted on positively charged slides, and coverslipped with antifade medium (Prolong Antifade Kit, Molecular Probes, Eugene, OR).

Analyses

RT-PCR—Cycle threshold values (CT values) were obtained from the linear region of baseline adjusted amplification curves. Each PCR plate contained a dilution series for both TIP39 and GAPDH whose CT values provided standard curves to calculate the amount of cDNA in the samples. Statistical analyses (Prism 4 for Windows, GraphPad Software, Inc., US) were performed by One-way Analysis of Variance to test age-dependency of mRNA levels, and by Two-way Analysis of Variance to test gender and gonadectomy effects at PND-300 in a separate experiment. Bonferroni Post-Tests were used for posthoc comparisons.

Histology—Sections were examined and images captured at 1300 × 1030 pixel resolution with a Photometrix CoolSnap Fx digital camera on an Olympus IX70 light microscope equipped with fluorescent epi-illumination using a 4 × objective. Contrast and sharpness were adjusted using the “levels” and “sharpness” commands in Adobe Photoshop CS 8.0. Full resolution was maintained until the photomicrographs were printed, at which point images were adjusted to a resolution of 300 dpi.

RESULTS

TIP39 mRNA expression in male and female rats

In situ hybridization histochemistry demonstrated that, as previously described for young adult male brains (Dobolyi et al., 2003b; Dobolyi et al., 2002), TIP39-expressing cells are present in both male and female brains in two regions: the subparafascicular area in the caudal thalamus and the medial paralemniscal nucleus in the lateral pons (Fig. 1). While the density of TIP39 cells varied with age and gender as described later, their localization was the same in male and female at all ages examined. In the caudal thalamus, TIP39-containing cells first appeared rostrally dorsolateral to the third ventricle at the most caudal level of the dorsomedial hypothalamic nucleus close to the midline. More caudally, TIP39 cells are shifted gradually more dorsally. Midway through their rostro-caudal extent, the TIP39 cells are situated medial to the fasciculus retroflexus, extending into the periventricular gray of the thalamus in male (Fig. 2) as well as female (Fig. 3) and disappearing caudally in the ventral portion of the most rostral part of the periaqueductal gray. A few cells appear ventrally to the main cell group and are aligned immediately next to the caudal end of the third ventricle (Figs. 2, 3). Some other cells are situated more laterally among the cells of the magnocellular as well as the parvocellular subparafascicular nuclei. We refer to all of these cells as the subparafascicular TIP39 cells because they occupy the subparafascicular area in and around the subparafascicular nucleus. In the lateral pons of male as well as female rats, TIP39 cells are located medial to the lateral lemniscus, and immediately dorsal to the rubrospinal tract at all ages examined (Figs. 1, 4). These cells occupy the ventral, caudal portion of the medial paralemniscal nucleus just rostral to the Kölliker-Fuse nucleus. TIP39-

containing cells have a cone-like distribution in this area, with the cells more scattered caudally.

TIP39 mRNA expression during postnatal development

The *in situ* hybridization signal for TIP39 increases in both the subparafascicular area (Figs. 2, 3) and the medial paralemniscal nucleus from PND-1 to PND-7 and then gradually decreases after PND-14. TIP39 mRNA levels are markedly reduced by PND-125 in both the subparafascicular area (Figs. 2, 3) and the medial paralemniscal nucleus (Fig. 4). Quantitative RT-PCR confirmed that the amount of TIP39 mRNA in the subparafascicular area decreases between PND-14 and -33 (Fig. 5A). TIP39 mRNA expression continues to decrease and is very low by PND-125 irrespective of the estrus cycle stage of the animals (not shown). Quantitative PCR also confirmed a similar pattern of decrease over time in the amount of TIP39 mRNA in the medial paralemniscal nucleus (Fig. 5B). The level of GAPDH mRNA is much greater than that of TIP39, probably because it is expressed in all of the dissected cells while TIP39 is expressed by a very small fraction. The GAPDH level increases between PND-1 and PND-7 but does not change after that, and does not depend upon gender (Fig 5C,D).

Sexual dimorphism in TIP39 mRNA levels

In young animals, we observed no significant difference between the TIP39 expression level of male and female rats using *in situ* hybridization histochemistry. We observed (see Results above) a decrease in TIP39 mRNA expression, which is continuous between PND-14 and PND-125. The decrease is greater in males, which results in greater mRNA expression of TIP39 in adult females than males. This result is quite dramatic, as visualized by *in situ* hybridization histochemistry in both the subparafascicular area (Figs. 2F,3F and 6A,C) and the medial paralemniscal nucleus (Fig. 4C,F). At PND-125 and PND-300, large gender differences were apparent in the TIP39 hybridization signals. Many neurons containing TIP39 mRNA can be visualized in females at PND-125 and PND-300 but only very few have detectable levels in the male at these ages, and those cells have low signal intensity.

In accordance with the results of the *in situ* hybridization histochemistry experiments, the quantitative PCR data also indicate difference between males and females in the amount of TIP39 expressed in both the subparafascicular area and the medial paralemniscal nucleus at PND-125 (Fig. 5A,B) and PND-300 (Fig. 7).

The effect of gonadectomy on TIP39 mRNA levels

The dramatic difference between male and female TIP39 mRNA expression that develops around the time of sexual maturation suggests that TIP39 synthesis may be regulated by gonadal hormones. However, castration and ovariectomy performed at PND-55 had no significant effect on TIP39 mRNA levels at PND-300, measured by *in situ* hybridization histochemistry (not shown) and by real-time RT-PCR (mRNA levels in the castrated male group were $117 \pm 33\%$ of the control group in the subparafascicular area and $116 \pm 32\%$ in the medial paralemniscal nucleus; mRNA levels in the ovariectomized female group were $95 \pm 23\%$ of the control group in the subparafascicular area and $86 \pm 24\%$ in the medial paralemniscal nucleus).

In contrast, pre-pubertal gonadectomy did have a significant effect on TIP39 mRNA expression. Gonadectomy performed at PND-24 resulted in greater expression of TIP39 mRNA at PND-300 in castrated as compared to control males (Fig. 6A,B) and in ovariectomized females as compared to control females (Fig. 6C,D). Real-time RT-PCR measurements confirmed the effects of pre-pubertal gonadectomy and showed an approximately 2-fold increase in the subparafascicular area (Fig. 7A) and about a 3-fold

increase in the medial paralemniscal nucleus in response to pre-pubertal gonadectomy (Fig. 7B). The pre-pubertal gonadectomy did not completely reverse the age-dependent decrease of TIP39 mRNA expression. The elevated level remained less than half of the TIP39 mRNA level at PND-14.

TIP39-immunoreactive cell bodies during postnatal development

We previously showed that an antiserum to TIP39 labels cells with the same distribution in the young adult male rat as demonstrated for TIP39 mRNA by *in situ* hybridization (Dobolyi et al., 2003b; Dobolyi et al., 2002). To test whether the large developmental and gender differences observed in TIP39 mRNA expression are reflected in peptide levels we examined the distribution of TIP39-immunoreactive cell bodies at several stages of postnatal development. Our results show that the distribution of TIP39-immunoreactive cell bodies is the same as that of TIP39 mRNA-containing cells in females as well as males and the developmental pattern of the intensity of immunolabeling is similar to that of TIP39 mRNA expression. TIP39 immunoreactivity is present in cell bodies of the subparafascicular area and the medial paralemniscal nucleus at PND-1 but its level is relatively low, as shown for the subparafascicular area in male (Fig. 8). The amount of labeling increases from PND-1 to PND-14 and then remains high until PND-33 (Fig. 8). Subsequently, TIP39 peptide level in cell bodies decreases and is very low at PND-125 (Fig. 8) and PND-300 (not shown). Similarly to TIP39 mRNA levels, TIP39 peptide levels in cell bodies of animals at PND-125 and PND-300 is also higher in female (not shown).

TIP39-immunoreactive fibers during postnatal development

TIP39 labeling is not detectable in fibers at PND-1 in either male (Fig. 9A) or female (Fig. 9G). The first labeling detected in fibers appeared at PND-7 in both genders, as demonstrated for the area of the paraventricular hypothalamic nucleus (Fig. 9B,H). The density of fibers further increases by PND-14. Immunolabeled TIP39 fibers were present in many brain areas between PND-14 and PND-57 in male as well as in female. There is no obvious difference between the intensity of TIP39 immunoreactivity of males and females at these ages. Furthermore, we observed no differences between these distributions and the one previously reported (Dobolyi et al., 2003b) in young adult male rats (around PND-57). In male as well as in female at PND-57, a very high density of TIP39 fibers was present in the medial prefrontal cortex, the lateral septum, the amygdalo-striatal transitional zone, the bed nucleus of the stria terminalis, the hypothalamic dorsomedial, paraventricular, and arcuate nuclei, the ectorhinal cortex, the inferior colliculus, and the parabrachial nuclei. In addition, the presence of abundant TIP39 fiber networks in many brain areas can be better appreciated at PND-33 than reported previously in 250–350 g rats (Fig. 10). A very high density of TIP39 fibers was present at PND-33 in the preoptic area including the medial preoptic nucleus (Fig. 10A), the posterodorsal medial amygdaloid nucleus (Fig. 10B), the amygdalohippocampal transitional zone (Fig. 10C), the lateral part of the periaqueductal gray (Fig. 10D), the ventral premamillary nucleus (Fig. 10E), and the ventral subiculum (Fig. 10F).

The density of TIP39-immunoreactive fibers decreased markedly by PND-125 in all brain areas containing TIP39 fibers as demonstrated in the paraventricular hypothalamic nucleus (Fig. 9). The density of TIP39-immunoreactive fibers remained low at PND-300 (not shown). The sexual dimorphism in TIP39 mRNA expression and immunoreactivity in cell bodies is also reflected in the peptide content of fibers at PND-125 and PND-300. The density of TIP39-immunoreactive fibers and the intensity of their immunolabeling is decreased at PND-125 (Fig. 9I) and PND-300 (not shown), but TIP39-positive fibers remain much more obvious in females.

PTH2 receptor levels during postnatal development

We did not observe differences in the location of PTH2 receptor expression between male and female rats. Furthermore, PTH2 receptor mRNA (not shown) and peptide levels, as shown in the lateral septal nucleus (Fig. 11), do not depend on age or gender as described above for TIP39.

DISCUSSION

The major finding of this study is that TIP39 expression peaks when rats are approximately 2 weeks old and that it undergoes a subsequent dramatic decline reaching low levels by PND-125. The decline is greater in males than females, resulting in significantly more TIP39 expression in older females than males. This suggests that TIP39 may play a role in sexual maturation and reproductive or other sexually dimorphic functions.

Methodological considerations

Observed changes in the *in situ* hybridization signal for TIP39 at different rat ages indicate that the amount of TIP39 mRNA in a single 12 μ m thick section changed during postnatal development and sexual maturation. Since individual cells are visualized it is apparent that the number of cells that express detectable amounts of mRNA and the amount per cell changed in parallel. The amount of TIP39 mRNA increased somewhat from PND-1 to PND-7 and decreased after PND-14 becoming very low by PND-125. Since the signal increased during a period when the brain is expanding relatively rapidly (Fuller and Geils, 1972) it is unlikely that brain size changes contribute to the observed differences. In contrast to the signal from individual cells, real-time RT-PCR measures mRNA levels in the total tissue dissected. Our *in situ* hybridization results show that TIP39 cells within the “upper brainstem” comprised of hypothalamus, thalamus and midbrain are restricted to the subparafascicular area. Therefore, TIP39 mRNA in the “upper brainstem” dissection is only derived from TIP39 cells in the subparafascicular area. Similarly, TIP39 mRNA in the pons is only derived from TIP39 cells in the medial paralemniscal nucleus. To reduce variation in RT-PCR experiments, we used GAPDH in multiplex PCR reactions to normalize the data and expressed the amount of TIP39 mRNA as the ratio to GAPDH mRNA. Also, to reduce variation during the reverse transcription (RT) step, we always measured RNA concentrations and performed the RT reaction with 2 μ g total RNA. Since GAPDH is expressed in all brain cells in about the same amount, the amount of GAPDH mRNA in 2 μ g total RNA does not depend on the size of the dissection. The amount of TIP39 mRNA in 2 μ g total RNA and so the ratio of TIP39 to GAPDH mRNA, on the other hand, depends on the size of a dissection, therefore, we took extra care to reproducibly dissect the tissue. In fact, this and to include all TIP39 cells with certainty, are the reasons why we chose large dissections. We found no differences in the amount of GAPDH mRNA in 2 μ g total RNA except a somewhat lower level at PND-1. The lower GAPDH mRNA level at birth could explain why the ratio of TIP39 to GAPDH mRNA is not smaller at PND-1 than PND-7 despite a lower TIP39 *in situ* hybridization signal at PND-1.

The variability of immunolabeling and the difficulties in its quantitative analysis limit immunocytochemistry as a quantitative technique to measure peptide levels. To reduce problems of reproducibility, we processed tissues the same way and always performed immunolabelings (and *in situ* hybridizations) for comparisons together. Furthermore, we used fluorescent labeling, where the signal can change with the amount of antigen better than with DAB visualization. That way, the large changes in TIP39 peptide level that occur during postnatal development and maturation, as well as the differences that exist between mature males and females, were detected as changes in immunoreactivity both in cell bodies and in fibers.

Changes in TIP39 in the developing brain

TIP39 is expressed by birth in both the subparafascicular area and the medial paralemniscal nucleus. TIP39 mRNA and peptide levels in cell bodies increase in the first week after birth. In contrast, TIP39 peptide is not detectable in fibers and terminals at PND-1 and its level is still low at PND-7. Because we visualized the fibers of TIP39 cells only by TIP39 immunolabeling, it is not possible to tell from our data if the projections from these cells exist before PND-7 and that TIP39 only appears in them at this age, or whether these fibers grow during the first postnatal week. TIP39 mRNA levels start to decrease after PND-14, and continue to decrease until PND-125. After that, the low level of TIP39 mRNA expression persists. Changes in TIP39 peptide levels in cell bodies and especially in fibers lag behind the changes in mRNA. The density of TIP39-immunoreactive fibers and the intensity of immunolabeling are still relatively high at PND-57. This may be significant because the brain functions of TIP39 cells are probably related to the presence of TIP39 in fibers and terminals. There is currently no information on the turnover rate or stability of TIP39 mRNA or peptide, and we do not know the relative sensitivity of our *in situ* hybridization and immunohistochemistry techniques. The density of TIP39-immunoreactive fibers and the intensity of their TIP39-immunolabeling are decreased dramatically by PND-125.

The time courses of postnatal changes in TIP39 levels are similar for the subparafascicular area and the medial paralemniscal nucleus suggesting that the same underlying mechanism is responsible for the changes in both TIP39-expressing brain regions. Our data provide no information regarding whether TIP39 cells degenerate during late postnatal development or whether their TIP39 expression decreases. We have not yet identified any independent markers for these neurons. However, the finding that brain PTH2 receptor levels do not change significantly during late postnatal development suggests that TIP39 can exert its actions if it reappears. Therefore, it is tempting to speculate that TIP39 cells remain intact and that TIP39 is induced in them in response to specific physiological stimuli.

Gender differences in TIP39 expression

There is no gender difference in TIP39 mRNA expression in young animals. As the level of TIP39 mRNA decreases with age, it becomes sexually dimorphic. A tendency for higher levels in females appears at PND-33. At PND-125 and PND-300, TIP39 mRNA expression is significantly higher in female than in male rats but even the female levels are decreased as compared to younger animals. Similar to TIP39 mRNA levels, there is a sexual dimorphism in TIP39 peptide level at this age. This gender difference is probably not due to particular estrus cycle stage of the female rats because each stage of the estrus cycle was relatively evenly represented in the PND-57 and PND-125 female groups. Similarly to the age related decline in TIP39 mRNA levels, the decrease in TIP39 peptide levels between PND-33 and PND-125 is also greater in males resulting in higher remaining immunoreactivity in cell bodies as well as fibers in females than in males. The difference in TIP39 expression between mature adult males and females must not be directly related to their gonadal steroids levels because gonadectomy did not affect TIP39 levels when performed in the adult. However, gonadectomy performed at PND-24 partially reversed the decrease in TIP39 levels suggesting that the decrease is related to sexual steroid hormonal effects occurring after PND-24. Consistent with this suggestion is the marked increase of sexual steroid hormone levels between PND-24 and PND-80 (Lamming, 1994). The gender difference, on the other hand, did not decrease in gonadectomized animals suggesting it is set up by events prior to PND-24.

Potential functions of TIP39

Previous studies suggest that TIP39 neurons might be involved in nociceptive processing (Dobolyi et al., 2002; LaBuda and Usdin, 2004), auditory functions (Dobolyi et al., 2003a; Palkovits et al., 2004), emotional changes (LaBuda et al., 2004), and endocrine regulation (Sugimura et al., 2003; Usdin et al., 2003; Ward et al., 2001). Areas that contain TIP39 neurons are involved in sexual (Coolen et al., 2004; Holstege et al., 2003) and maternal functions (Li et al., 1999; Lin et al., 1998), suggesting that TIP39 may be as well. Nociception (Hamm and Knisely, 1988), hearing (Geal-Dor et al., 1993), endocrine functions (Eden, 1979; Moguilevsky and Wuttke, 2001) and clearly sexual functions (Lamming, 1994), undergo profound postnatal development in the rat. Bearing in mind that TIP39's homologue, PTHrP, has well-established effects on the development of many tissues (Kronenberg et al., 1998) and that TIP39 can affect the proliferation of some cell types *in vitro* (Misiano et al., 2003), the transient expression of TIP39 during postnatal development suggests that this peptide may be involved in developmental aspects of the above mentioned brain functions. Our data also point to the importance of the age of the experimental animals when designing future functional experiments on the role of TIP39.

The peak and the timing of the decrease in TIP39 levels best correlates with sexual maturation. Solicitation, pacing and lordosis in females (Erskine, 1989), and genital grooming, erection, mounting, intromission, and ejaculation in males (Sachs and Meisel, 1988) appear as typical sexual behaviors in rat during puberty, which occurs between postnatal days 30–50 in rat (Ebling and Cronin, 2000). Hormonal and other physiological changes take place in parallel with the behavioral events. The body weight of the rats is steadily increasing. Elevated plasma sex steroid, growth hormone and luteinizing hormone levels are reached during puberty (Hull and Harvey, 2002; Lamming, 1994). In contrast, leptin levels decrease after sexual maturation in a sexually dimorphic manner: they reach low levels in female and very low levels in male (Caprio et al., 2001). There is an interconnected network of brain centers that participate in the coordination and central regulation of these behavioral and physiological changes. Many of these centers are activated during particular sexual and/or maternal behaviors similarly to how the lateral part of the subparafascicular area is activated following male ejaculation (Coolen et al., 2004; Holstege et al., 2003) or the medial part of the subparafascicular area (Lin et al., 1998) are activated in lactating females. Centers of brain reproductive circuitry often have sexually dimorphic features (Gorski, 1985; Shah et al., 2004; Simerly, 2002), which is consistent with the fundamentally different roles of female and male reproductive circuits. It has been suggested that many limbic, preoptic and hypothalamic areas, including the medial prefrontal cortex, the lateral septum, the bed nucleus of the stria terminalis, the medial preoptic nucleus, the anteroventral periventricular, paraventricular and ventromedial hypothalamic nuclei, the arcuate nucleus, the medial and central amygdaloid nuclei, the amygdalo-hippocampal transitional zone, the ventral premammillary nucleus, the ventral subiculum, and the periaqueductal gray are part of brain reproductive circuitry (Finn et al., 1993; Gammie and Nelson, 2001; Lonstein et al., 1998; Numan and Sheehan, 1997; Sachs and Meisel, 1988; Shah et al., 2004; Sheehan et al., 2000; Simerly and Swanson, 1986; Veening and Coolen, 1998). Based on the time course of the decrease of TIP39 expression, the sexually dimorphic TIP39 content of the adult brain, the distribution of TIP39 fibers, and the afferent connections of brain regions expressing TIP39 (Wang et al., 2006a), we hypothesize that TIP39 may play a role in certain aspects of central reproductive regulation.

In conclusion, we demonstrated that TIP39 level peaks around PND-14 during postnatal development in male as well as female rats, and gradually decreases afterwards. A significant amount of TIP39 is still present at PND-57. At PND-125 and PND-300, TIP39 levels are further decreased in a sexually dimorphic manner. While a significant amount of TIP39 remains in females, the level of TIP39 in males at these ages is very low. These

results will promote investigation of TIP39's role in functions with similar developmental time course and sexual dimorphism.

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TABLE OF ABBREVIATIONS USED IN THE FIGURES

ac	anterior commissure
cc	corpus callosum
DG	dentate gyrus
DR	dorsal raphe nucleus
IC	inferior colliculus
ICE	inferior colliculus, external cortex
f	fornix
fr	fasciculus retroflexus
H	hippocampus
ic	internal capsule
ll	lateral lemniscus
LV	lateral ventricle
ml	medial lemniscus
MR	mamillary recess of the third ventricle
mt	mammillothalamic tract
ot	optic tract
PH	posterior hypothalamic nucleus
Pir	piriform cortex
PnO	pontine reticular nucleus, oral part
PTH	parathyroid hormone
py	pyramidal tract
rs	rubrospinal tract
SPF	subparafascicular area
TIP39	tuberoinfundibular peptide of 39 residues
VB	ventrobasal thalamic nucleus
3V	third ventricle
5th	root of the trigeminal nerve

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Fig. 1.

The localization of the two major groups of TIP39-expressing cell bodies in male as well as female rat brain. Arrows point to the location of TIP39-expressing brain regions on a schematic diagram. Micrographs showing labeled cells in these areas are shown in subsequent figures, and the distribution in young males has been described in detail (Dobolyi et al., 2003b; Dobolyi et al., 2002). **A** - Subparafascicular area in the caudal thalamus. **B** - Medial paralemniscal nucleus in the lateral pons.

Fig. 2. TIP39 expression in the subparafascicular area during postnatal development in male rat. Dark field images following radioactive *in situ* hybridization demonstrate an increase of TIP39 mRNA levels between PND-1 and PND-14 and a continuous marked decrease between PND-14 and -125. **A** - PND-1. **B** - PND-7. **C** - PND-14. **D** - PND-33. **E** - PND-57. **F** - PND-125. Scale bar = 200 μ m.

Fig. 3. TIP39 expression in the subparafascicular area during postnatal development in female rat. *In situ* hybridization demonstrates an increase of TIP39 mRNA levels between PND-1 and PND-14 and a continuous slight decrease between PND-14 and -125. **A** - PND-1. **B** - PND-7. **C** - PND-14. **D** - PND-33. **E** - PND-57. **F** - PND-125. Scale bar = 200 μ m.

Fig. 4.

TIP39 expression in the medial paralemniscal nucleus in male and female during postnatal development. *In situ* hybridization images of male (**A, B, C**) and female (**D, E, F**) rats demonstrate sexual dimorphism in the amount of TIP39 mRNA at PND-125. **A, D**-PND-7. **B, E** - PND-33. **C, F** - PND-125. Scale bar = 200 μ m.

Fig. 5.

TIP39 expression during postnatal development measured by quantitative RT-PCR. **A, B** - TIP39 mRNA levels are expressed as the ratio to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the subparafascicular area (**A**) and the medial paralemniscal nucleus (**B**), respectively. The levels of TIP39 mRNA decreased over time, tested by One-way Analysis of Variance ($p < 0.01$). Bonferroni's Multiple Comparison Tests indicated significant differences ($p < 0.05$) between postnatal day 14 and 125. In addition, TIP39 mRNA levels seem to be higher in the older females than in the older male animals (this effect achieved statistical significance at older ages, see Fig 6). GAPDH expressed as a ratio to total RNA does not change during development either in the subparafascicular area (**C**) or the medial paralemniscal nucleus (**D**).

Fig. 6.

In situ hybridization demonstrates the effect of gonadectomy performed at PND-24 on TIP39 expression in the subparafascicular area at PND-300. Dark field images demonstrate an increase in TIP39 mRNA levels in response to gonadectomy. **A** - control male. **B** - castrated male. **C** - control female. **D** - ovariectomized female. Scale bar = 300 μ m.

Fig. 7.

The effect of PND-24 gonadectomy on TIP39 expression at PND-300. **A** -subparafascicular area. **B** - medial paralemniscal nucleus. TIP39 mRNA levels were measured by quantitative RT-PCR. TIP39 mRNA levels are normalized to GAPDH. TIP39 levels significantly increased in both sites in response to castration, tested with Two-way Analysis of Variance ($p < 0.01$). Bonferroni Post-Tests indicated significant differences ($p < 0.05$) between male and female controls in the subparafascicular area.

Fig. 8. TIP39-immunoreactive cell bodies in the subparafascicular area during postnatal development in the male rat. Sections are labeled using fluorescent amplification immunocytochemistry. **A** - PND-1. **B** - PND-7. **C** - PND-14. **D** - PND-33. **E** - PND-57. **F** - PND-125. Scale bar = 500 μ m.

Fig. 9.

TIP39-immunoreactive fibers in the area in and around the paraventricular hypothalamic nucleus during postnatal development. Male (A–F) and female (G–L) rat sections were labeled with fluorescent amplification immunocytochemistry. A - PND-1 male. B - PND-7 male. C - PND-14 male. D - PND-33 male. E - PND-57 male. F - PND-125 male. G - PND-1 female. H - PND-7 female. I - PND-14 female. J - PND-33 female. K - PND-57 female. L - PND-125 female. The borders of the paraventricular hypothalamic nucleus are indicated with dashed white lines. When TIP39 immunoreactive fibers are present, their density is highest within the paraventricular hypothalamic nucleus. TIP39-immunoreactive fibers appear by PND-7. TIP39 immunoreactivity is increased by PND-14 and remains high until PND-57. At PND-125, TIP39 immunoreactivity is decreased. The decrease is more pronounced in male resulting in more intense TIP39 immunolabeling in female (L) than in male (F) at PND-125. Scale bar = 400 μ m.

Fig. 10.

TIP39-immunoreactive fibers in brain areas containing a high density of TIP39 fibers in 33 days old male rats. Sections are labeled using fluorescent amplification immunocytochemistry. Arrows point to the following brain areas: **A** - medial preoptic nucleus. **B** - posterodorsal medial amygdaloid nucleus. **C** - amygdalohippocampal transitional zone. **D** - periaqueductal gray, lateral part. **E** - ventral premamillary nucleus. **F** - ventral subiculum. Scale bars = 500 μ m.

Fig. 11. PTH2R-immunoreactive cell bodies and fibers in male and female lateral septum during postnatal development. Sections labeled using fluorescent amplification immunocytochemistry demonstrate that PTH2 receptor immunoreactivity does not show dependency on gender or age. **A** - PND-57 male. **B** - PND-125 male. **C** - PND-300 male. **D** - PND-57 female. **E** - PND-125 female. **F** - PND-300 female. Scale bar = 500 μ m.