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The TIP39-PTH2 receptor system: unique peptidergic cell groups in the brainstem and their interactions with central regulatory mechanisms

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Abstract

Tuberoinfundibular peptide of 39 residues (TIP39) is the recently purified endogenous ligand of the previously orphan G-protein coupled parathyroid hormone 2 receptor (PTH2R). The TIP39-PTH2R system is a unique neuropeptide-receptor system whose localization and functions in the central nervous system are different from any other neuropeptides. TIP39 is expressed in 2 brain regions, the subparafascicular area in the posterior thalamus, and the medial paralemnisal nucleus in the lateral pons. Subparafascicular TIP39 neurons seem to divide into a medial and a lateral cell population in the periventricular gray of the thalamus, and in the posterior intralaminar complex of the thalamus, respectively. Periventricular thalamic TIP39 neurons project mostly to limbic brain regions, the posterior intralaminar thalamic TIP39 neurons to neuroendocrine brain areas, and the medial paralemnisal TIP39 neurons to auditory and other brainstem regions, and the spinal cord. The widely distributed axon terminals of TIP39 neurons have a similar distribution as the PTH2R-containing neurons, and their fibers, providing the anatomical basis of a neuromodulatory action of TIP39. Initial functional studies implicated the TIP39-PTH2R system in nociceptive information processing in the spinal cord, in the regulation of different hypophysiotropic neurons in the hypothalamus, and in the modulation of affective behaviors. Recently developed novel experimental tools including mice with targeted mutations of the TIP39-PTH2R system and specific antagonists of the PTH2R will further facilitate the identification of the specific roles of TIP39 and the PTH2R.

Keywords

neuropeptide; subparafascicular; posterior intralaminar thalamic; limbic functions; endocrine hypothalamic regulations; nociceptive action; paralemnisal; auditory brainstem

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1 Introduction

Tuberoinfundibular peptide of 39 residues (TIP39) was identified on the basis of its activation of the parathyroid hormone 2 receptor (PTH2R), a seven transmembrane domain G-protein coupled receptor (Usdin *et al.*, 1999b). The distribution of TIP39 containing fibers and terminals is very similar to the distribution of PTH2R-containing neurons and neuronal fibers throughout the brain (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007), and TIP39 is a potent and selective PTH2R agonist (Usdin, 2000). This functional and anatomical match suggests that TIP39 is the endogenous ligand of the PTH2R in the brain, and that they form a neuromodulator system. TIP39 neurons have a highly restricted localization. This pattern of synthesis by cells in a few discrete areas and widespread, but still topographically organized, projections to several distant brain areas resembles several other recently developed neuropeptide systems including, for example, relaxins (Ma and Gundlach, 2007), orexins (Baumann and Bassetti, 2005), calcitonin-gene related peptide (van Rossum *et al.*, 1997), prolactin-releasing peptide (Roland *et al.*, 1999), kisspeptin (Mikkelsen and Simonneaux, 2009), and urocortins (Pan and Kastin, 2008). Based on the available data, however, the TIP39-PTH2R system is a unique neuropeptide-receptor system whose localization and functions in the central nervous system are different from any other neuropeptides.

TIP39 is a member of a small peptide family comprised of parathyroid hormone (PTH), parathyroid hormone-related peptide (PTHrP) and TIP39 (Usdin *et al.*, 1999b). Mature PTH and PTHrP are polypeptides of about 100 residues. They are products of separate genes but they activate the parathyroid hormone 1 receptor (PTH1R) with equal potency (Gensure *et al.*, 2005; Muff *et al.*, 1994). Their first 34 or 36 residues are sufficient for high-affinity binding and full efficacy at the PTH1R, and they share 12 of these amino acids (Gillespie and Martin, 1994; Martin *et al.*, 1991). The primary sequence of TIP39 is quite different. It contains only four of the residues that are common to PTH(1–34) and PTHrP(1–36), and similar residues at several additional positions (Usdin *et al.*, 1999b). However, TIP39 has a backbone structure that can be nearly superimposed on that of PTH (Piserchio *et al.*, 2000). Based on the similarity of TIP39 to PTH and its activation of the PTH2R, TIP39 is referred to as parathyroid hormone 2 in the UniGene database at <http://www.ncbi.nlm.nih.gov/sites/entrez> (Mm.207078 for the mouse and Hs.339845 for the human gene). However, this name is also used for a second form of PTH found in fish that more closely resembles mammalian PTH than does TIP39 (Gensure *et al.*, 2004). PTH, produced by the parathyroid gland, is the most important regulator of calcium homeostasis. It increases plasma calcium ion concentration *via* direct actions in the kidney and the skeleton (Hurwitz, 1996; Rizzoli *et al.*, 1992). PTHrP is a paracrine factor that functions in a number of organs and plays a critical role in skeletal development (Law *et al.*, 1994; Martin *et al.*, 1997). TIP39 and the PTH2R are expressed at very low levels in kidney and bone (Usdin *et al.*, 1996; Usdin *et al.*, 1999a). In the periphery, they may play a role in the cardiovascular system (Eichinger *et al.*, 2002; Ross *et al.*, 2005; Ross *et al.*, 2007) and in gonadal function (Usdin *et al.*, 2008). However, TIP39 and the PTH2R are most abundant in the central nervous system (Dobolyi *et al.*, 2002; Usdin *et al.*, 1995).

Since the last review on the TIP39-PTH2R system (Usdin *et al.*, 2003), the cell groups expressing TIP39 and the PTH2R as well as their neuronal connections have been identified. TIP39 and the PTH2R have unique expression patterns in the brain, which has facilitated the description of previously unrecognized anatomical structures and connections. TIP39 is expressed in only three sites in the brain, the medial and lateral parts of the subparafascicular area in the caudal thalamus, and the medial paralemnsical nucleus in the lateral pons (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007). Widespread connections of the subparafascicular area implicate this region in the regulation of limbic and endocrine functions (Dobolyi *et al.*, 2003a; Wang *et al.*, 2006b, c). Projections from the medial paralemnsical nucleus to a number of auditory areas, as well as some hypothalamic and viscerosensory regions, suggest that this

newly identified nucleus may play a role in auditory and nociceptive processes (Dobolyi *et al.*, 2003a; Dobolyi *et al.*, 2002; Varga *et al.*, 2008). The distribution of PTH2R containing neurons and fibers (Bagó *et al.*, 2009; Faber *et al.*, 2007; Wang *et al.*, 2000) is consistent with these proposed functions. In fact, the subregional similarities in the locations of TIP39 and PTH2R terminals (Dobolyi *et al.*, 2006a; Faber *et al.*, 2007), together with the demonstration of the glutamatergic nature of PTH2R terminals in the hypothalamus (Bagó *et al.*, 2009; Dobolyi *et al.*, 2006a), lead us to suggest an axo-axonic mechanism of action for the TIP39-PTH2R neuromodulator system. Embryonic (Brenner *et al.*, 2008) and postnatal development (Dobolyi *et al.*, 2006b) of the TIP39-PTH2R system has also been described recently. These data support the separation of the medial and lateral subparafascicular TIP39 neurons into separate groups (Brenner *et al.*, 2008), and reveal a decline in TIP39 but not PTH2R levels during the period of sexual maturation (Dobolyi *et al.*, 2006b). Initial functional studies implicate TIP39 in the regulation of the release of pituitary hormones (Sugimura *et al.*, 2003; Ward *et al.*, 2001), affect-related behaviors (Fegley *et al.*, 2008; LaBuda *et al.*, 2004), and the modulation of some aspects of nociceptive signaling (Dobolyi *et al.*, 2002; LaBuda and Usdin, 2004). Furthermore, studies using *c-fos* to identify activated neurons suggest that TIP39 neurons are involved in sexual function (Wang *et al.*, 2006a) and the audiogenic stress response (Palkovits *et al.*, 2004). Recently developed novel experimental tools including selective antagonists (Kuo and Usdin, 2007; Visegrady *et al.*, 2007) and transgenic animals (Faber *et al.*, 2007; Fegley *et al.*, 2008; Usdin *et al.*, 2008) are likely to further facilitate investigation of the functions of the TIP39-PTH2R system.

2 Peptide and gene structure of tuberoinfundibular peptide of 39 residues (TIP39)

2.1 Purification and sequencing of TIP39

Initial studies of the human PTH2R showed that it is activated by PTH (Usdin *et al.*, 1995). However, Usdin did not detect synthesis of PTH in the rat brain, and found activity that activated the PTH2R and not the PTH1R in bovine hypothalamus (Usdin, 1997). The rat PTH2R is poorly activated by PTH, and bovine hypothalamic extracts contain activity that is more potent and more efficacious at the rat PTH2R than PTH, based on stimulation of cAMP accumulation in cultured cells transfected with the PTH2R (Hoare *et al.*, 1999). This PTH2R-selective ligand, with the sequence SLALADDAAFRERARLLAALERRHWLNSYMHKLLVLDAP, was purified from an acid extract of fifty pounds of bovine hypothalamus (Usdin *et al.*, 1999b). Mass spectrometry provided no evidence for posttranslational modification of any of the amino acids. Chemically synthesized and purified TIP39 had identical molecular weight and the same fragment ions were produced following trypsin digestion (Usdin *et al.*, 1999b).

2.2 Structure of TIP39

The structure of TIP39 in a membrane mimic detergent was elucidated by CD and NMR spectroscopy. Similar to PTH(1–34), TIP39 contains two stable alpha-helices at the N and C termini separated by a region of undefined structure (Piserchio *et al.*, 2000). The N-terminal helix shares a high structural and sequential homology with PTH (Pellegrini *et al.*, 1998), suggesting a similar function in receptor signaling. The structural differences between TIP39 and PTH(1–34) include the lengths and amphipathic character of the helices as well as the location of the flexible region between the helices (Piserchio *et al.*, 2000). A pronounced difference is that TIP39(Trp25) falls in the middle of the structurally unordered region in contrast to a well defined helical portion observed for the PTH(Trp23). The C-terminal helix of TIP39 has some charged residues on the hydrophobic face, reducing the amphipathic nature of the helix in comparison with that of PTH(1–34). This difference could diminish the importance of the interaction of the C terminus of TIP39 with the large extracellular N terminus

of the receptor, postulated to be an important step in recognition and binding of PTH to the PTH1R (Mierke *et al.*, 2007). Finally TIP39 places Asp7 in the key position corresponding to PTH(Ile5) and PTHrP(His5), which may contribute to the selectivity of TIP39 toward the PTH2R. NMR spectroscopy also revealed that TIP39 associates with the anionic membrane surface *via* its positively charged residues but does not insert into the membrane hydrophobic compartment (Mason *et al.*, 2005; Piserchio *et al.*, 2000). However, peptide accumulation at the membrane surface is not significantly increased by this interaction (Mason *et al.*, 2005).

2.3 Structure of the gene encoding TIP39

Following the purification of bovine TIP39, the mouse and human genes encoding TIP39 were identified in database searches based on sequence similarities (Dobolyi *et al.*, 2002; Hansen *et al.*, 2002; John *et al.*, 2002). The human TIP39 gene is on the long arm of chromosome 19 at band 19q13.3. Human and murine cDNAs were obtained by RACE-PCR and are available under the GenBank accession numbers: AY037555 and AC073740 (Hansen *et al.*, 2002) and AY048588 and AY048587 (John *et al.*, 2002). On alignment, the nucleotide sequences of the human and murine cDNAs have 80% identity. The cDNAs encoding both TIP39 precursors are rich in guanine and cytosine (GC-content: 74.3% and 69.7%, respectively) compared with a human genome-wide average of 41%. TIP39 cDNA (Fig. 1) consists of a 5'-untranslated sequence of 102 bp, an open reading frame of 300 bp, and 55 bp of 3'-untranslated sequence containing a polyadenylation signal (Hansen *et al.*, 2002; John *et al.*, 2002). The human and mouse genes contain two coding exons separated by an intron at corresponding sites. Exon 1 encodes 43 aa of the TIP39 precursor protein, exon 2 encodes the remaining 57 aa. The first 30 aa are predicted to function as a signal peptide, which is followed by a signal peptidase cleavage site (Dobolyi *et al.*, 2002; Hansen *et al.*, 2002). The TIP39 precursor also contains two possible dibasic cleavage sites (Arg-Arg motif with compatible adjacent residues). The first separates an intercalated peptide from the peptide that was purified, the second is found at position 22/23 within the TIP39 sequence (the 39 residue sequence that was purified is referred to as TIP39 and the predicted primary translation product as pre-pro-TIP39, the putative pro-TIP39 formed following signal peptide cleavage has not been demonstrated). Human and mouse pre-pro-TIP39 share 79% overall identity and there is 89% identity within the TIP39 peptide. Thirty-five out of 39 residues are identical, with changes in amino acid class at positions 24, 27, 31 and a change to a homologous residue at position 35. TIP39 sequences have the highest degree of identity in the N-terminal region, all 23 residues in the N-terminus are identical. There is 100% identity between the sequence of human and bovine TIP39 (Della Penna *et al.*, 2003). The rat TIP39 cDNA was also cloned and found to be very similar to that of the mouse (Della Penna *et al.*, 2003; Dobolyi *et al.*, 2002). The predicted amino acid sequences of mouse and rat TIP39 are identical (Della Penna *et al.*, 2003; Dobolyi *et al.*, 2002).

Sequences of peptides homologous to TIP39 are present in teleost species including the Japanese pufferfish fugu (*Takifugu rubripes*), the zebrafish (*Danio rerio*), the Nile tilapia (*Oreochromis niloticus*), and the euryhaline Mozambique tilapia (*Oreochromis mossambicus*) (Papasan *et al.*, 2004; Shoemaker *et al.*, 2006). The predicted processed peptide of fugu and zebrafish consists of 39 residues (Papasan *et al.*, 2004) while that of tilapia species consists of only 38 residues, corresponding to the first 38 amino acid of other species (Shoemaker *et al.*, 2006). The teleost TIP39 homologues have 81-83% nucleotide sequence identity and 97% amino acid sequence similarity with each other (Papasan *et al.*, 2004; Shoemaker *et al.*, 2006) and 57-63% sequence identity with the mammalian TIP39 homologs (Papasan *et al.*, 2004; Shoemaker *et al.*, 2006). Although teleosts also have an intercalated peptide in the precursor, its sequence is poorly conserved, as is the signal peptide. In addition, the intercalated peptide is much longer in teleosts (fugu TIP39: 72 amino acid residues; zebrafish TIP39: 91 amino acid residues) than the comparable mammalian sequence (31 amino acids). RACE-PCR

suggested that there could be variant zebrafish pre-pro-TIP39 sequences (GenBank accession numbers: AY307076 and AY306196), with a short variant initiated at the third Met instead of the first Met and thus lacking 63 nucleotides from the region encoding the poorly conserved signal peptide, but not missing any amino acids of the processed peptide. However, typical splice donor and acceptor sites were not found, thus it is possible that the putative splice variant is an artifact and the zebrafish is not different from other species where no splice variants of the TIP39 gene were identified (Papasani *et al.*, 2004).

3 TIP39 expressing cell groups

3.1 TIP39 expression in different organs

TIP39 expression appears abundant in several rat brain regions and the testis based on non-quantitative RT-PCR, and it is detectable in the eye and the dorsal root ganglia (Dobolyi *et al.*, 2002), kidney and pancreas (Eichinger *et al.*, 2002), and heart (Ross *et al.*, 2005). In human, TIP39 expression was detected by RT-PCR in the brain, trachea, fetal liver, kidney and heart (Hansen *et al.*, 2002). In tilapia species, RT-PCR showed TIP39 expression in kidney, heart, liver and testes (Shoemaker *et al.*, 2006). In the zebrafish, whole-mount *in situ* hybridization showed TIP39 mRNA in the brain and heart (Papasani *et al.*, 2004). Northern blots in mice showed a prominent message of approximately 4.5 kb in testis, which was also observed, at much lower intensity, in liver, kidney, and possibly heart (John *et al.*, 2002). Additional transcripts of about 1.5 kb and 1.0 kb were detected in these organs as well as in the brain (John *et al.*, 2002). The larger hybridizing RNA may be incompletely processed pre-mRNA.

The distribution of TIP39 expression within individual organs has been investigated by *in situ* hybridization in mouse testis (John *et al.*, 2002; Usdin *et al.*, 2008), and rat (Dobolyi *et al.*, 2003b; Dobolyi *et al.*, 2002), mouse (Faber *et al.*, 2007; John *et al.*, 2002), and macaque brain (Bagó *et al.*, 2009). In the testis, TIP39 mRNA is expressed in the epithelium of some but not all seminiferous tubules, indicating that it is expressed in a stage-specific manner (John *et al.*, 2002; Usdin *et al.*, 2008). Higher magnification images of TIP39 *in situ* hybridization suggest that most of the label is present over the middle third of the epithelium, the location of developing spermatogenic cells (Usdin *et al.*, 2008). Immunolabeling of TIP39, and visualizing beta-galactosidase activity in mice containing beta-galactosidase driven by the TIP39 promoter, confirmed the location of TIP39 in developing spermatogenic cells (Usdin *et al.*, 2008). TIP39-expressing neurons have a highly restricted localization in the brain of rodents (Dobolyi *et al.*, 2003b; Dobolyi *et al.*, 2002; Faber *et al.*, 2007; John *et al.*, 2002; Ky and Shughrue, 2002), macaque (Bagó *et al.*, 2008), and zebrafish (Papasani *et al.*, 2004) as described below.

3.2 TIP39 neurons in the posterior diencephalon

3.2.1 The subparafascicular area (SPF)—TIP39-expressing neurons are present in the subparafascicular nucleus and the surrounding subparafascicular area of the thalamus (Dobolyi *et al.*, 2002; John *et al.*, 2002), a relatively little studied area situated in the ventromedial part of the posterior thalamus (Faul and Mehler, 1985). The subparafascicular nucleus was originally described as a large area below the fasciculus retroflexus and the parafascicular nucleus (Rioch, 1929). Subsequently, the subparafascicular nucleus was separated into 2 nuclei. The magnocellular (also called medial) subparafascicular nucleus which has a cytoarchitectonically well-defined circular appearance on coronal sections from bregma levels -3.8 to -4.3 mm (Faul and Mehler, 1985; Paxinos and Watson, 2005). The parvocellular or lateral subparafascicular nucleus (SPFp), situated from bregma levels -4.2 to -4.8 mm has a caudo-laterally extended orientation above the medial lemniscus as far as the ventral part of the medial geniculate body (Ledoux *et al.*, 1987; Papez and Aronson, 1934). The subparafascicular nuclei have been considered thalamic nuclei with unknown function (Faul

and Mehler, 1985; Turner and Herkenham, 1991) or members of the posterior intralaminar group of nuclei (LeDoux *et al.*, 1985; Rub *et al.*, 2002). Most authors, however, do not consider them to be midline or intralaminar nuclei (Berendse and Groenewegen, 1991; Van der Werf *et al.*, 2002). The complex shape and the difficulty in defining the area contributed to the introduction of several different terminologies describing brain areas that include the subparafascicular nuclei or parts of them, as well as surrounding brain areas. The term 'subparafascicular area' and its 'compartments' corresponding to the magnocellular subparafascicular nucleus and the periventricular gray of the thalamus were introduced to describe connectional data (Moriizumi and Hattori, 1991, 1992). The area caudal and dorsal to the subparafascicular nucleus has also been called 'subfascicular area' (Peschanski and Mantyh, 1983). The term 'periventricular gray of the caudal thalamus' was used to describe the location of the A11 dopaminergic cells (Hökfelt *et al.*, 1984; Hökfelt *et al.*, 1979; Skagerberg and Lindvall, 1985), and to describe sites for stimulus-induced analgesia in the region (Boivie and Meyerson, 1982; Rhodes and Liebeskind, 1978).

TIP39 neurons are situated close to the midline in and around the magnocellular subparafascicular nucleus within the periventricular gray of the thalamus, as well as in the parvocellular (lateral) subparafascicular nucleus and the surrounding area above the medial lemniscus and further lateral, as far as the area ventromedial to the medial geniculate body (Fig. 2). Because the medially and laterally positioned TIP39 neurons seemed relatively contiguous on horizontal sections (Fig. 2D) they were initially considered a single cell group and the term subparafascicular area was introduced to describe the distribution of TIP39 neurons. However, recent developmental (Brenner *et al.*, 2008) and functional (Wang *et al.*, 2006a) data, described below, suggest that the periventricular and lateral subparafascicular/posterior intralaminar thalamic TIP39 neurons constitute separate cell groups, which makes the use of the unifying term subparafascicular area less plausible.

3.2.2 TIP39 neurons in the periventricular gray of the thalamus (PVG)—

Periventricular thalamic TIP39 neurons constitute the largest TIP39 cell group in the brain of young adult rats (Dobolyi *et al.*, 2003b) and mice (Faber *et al.*, 2007), with about 600-1000 neurons per side. TIP39 neurons in the PVG appear rostrally first above the third ventricle, close to the midline at bregma level -3.8 mm (Fig. 2A1). These TIP39 neurons are located ventral to the central median nucleus of the thalamus, dorsal to the posterior hypothalamic nucleus and medial to the parvocellular ventral posterior nucleus of the thalamus and mostly medial to the magnocellular subparafascicular nucleus (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007). More caudal TIP39 neurons are located more and more dorsally between the midline and the fasciculus retroflexus (Fig. 2A2). Midway through the rostrocaudal extent of the PVG, a few cells appear ventrally to the main cell group and are aligned immediately next to the caudal end of the third ventricle (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007). Additional TIP39 neurons are situated more laterally, ventral to the fasciculus retroflexus. Caudally, the density of TIP39 cells sharply decreases and the cells disappear as the PVG becomes the periaqueductal gray of the midbrain at the level of the posterior commissure without apparent cytoarchitectonic change (Fig. 2A3). In sagittal sections, the distribution of the periventricular TIP39 neurons has a sigmoid shape with a rostro-ventral to postero-dorsal orientation (John *et al.*, 2002; Wang *et al.*, 2006c) (Fig. 2B,C). These TIP39 neurons in the PVG are intermingled with tyrosine hydroxylase-containing neurons corresponding to the A11 dopaminergic cell group (Hökfelt *et al.*, 1979; Skagerberg and Lindvall, 1985). Although the distributions of TIP39 and dopaminergic cell bodies largely overlap, the dopaminergic cells are situated somewhat more laterally than the TIP39 cells. Furthermore, no TIP39/tyrosine hydroxylase double-labeled cells are present (Dobolyi *et al.*, 2003b; Wang *et al.*, 2006c).

3.2.3 TIP39 neurons in the posterior intralaminar complex of the thalamus (PIL)

—In the adult male rat, only about 200-300 relatively small, fusiform, and horizontally oriented

TIP39 neurons are visible per side between bregma levels -4.2 to -6.1 mm in the lateral cell group of the posterior thalamus. However, the number of TIP39 neurons is markedly higher in this area during embryonic (Brenner *et al.*, 2008) and early postnatal development (Dobolyi *et al.*, 2006b) as described below. The laterally positioned TIP39 neurons constitute a horizontal cell line in an area over the medial lemniscus and further laterally as far as the area ventromedial to the medial geniculate body (Figs. 2D, 3). The area over the medial lemniscus corresponds to the parvicellular subparafascicular nucleus (SPFp) while the area ventromedial to the medial geniculate body includes the posterior intralaminar thalamic nucleus (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007). In addition, some TIP39 neurons are situated below the lateral part of the medial lemniscus in the lateral territory of the caudal zona incerta between bregma levels -4.5 to 5.2 mm (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007). A similar caudo-laterally elongated topographical arrangement has been described previously and referred to as the posterior intralaminar complex of the thalamus (PIL) (LeDoux *et al.*, 1985). This term has recently been used to describe the position of the thalamic TIP39 neurons lateral to the fasciculus retroflexus (Brenner *et al.*, 2008; Dobolyi *et al.*, 2006a; Faber *et al.*, 2007). In addition, some studies may have included the brain area where the lateralmost TIP39 neurons are located in a region designated the mesencephalon (Shimura and Shimokochi, 1991) or referred to as the 'zona incerta/lateral tegmentum continuum' (Maillard and Edwards, 1991).

The rostro-medial to caudo-lateral arrangement of TIP39 neurons resembles the distribution of calcitonin-gene related-peptide (CGRP)-containing neurons in the posterior thalamus. CGRP neurons are also situated in the parvicellular part of the subparafascicular nucleus, the posterior intralaminar thalamic nucleus extending as far laterally as the area around the medial geniculate body (Dobolyi *et al.*, 2005; Ishida-Yamamoto and Tohyama, 1989; Kresse *et al.*, 1995; Skofitsch and Jacobowitz, 1985). It can be recognized in horizontal sections that CGRP neurons constitute a contiguous arch of cells in the posterior thalamus (Dobolyi *et al.*, 2005; Kruger *et al.*, 1988), that has been referred to as the 'CGRP nucleus' of the thalamus (Kruger *et al.*, 1988). The thalamic brain area corresponding to the region containing CGRP neurons around the medial geniculate body has also been unified by the term 'posterior paralaminar thalamic nuclei' due to their location adjacent to the internal medullary lamina, their similar auditory inputs and their pattern of projection to the cerebral cortex (Linke, 1999). In addition to the obvious similarities between the unique caudo-laterally extended distributions of TIP39 and CGRP in the posterior thalamus there are several significant differences. Although a few neurons are double labeled with TIP39 and CGRP in the lateral part of the posterior thalamus (Dobolyi *et al.*, 2003b), TIP39 neurons typically are not labeled by a CGRP antibody and are located medial to the bulk of CGRP neurons in coronal sections (Brenner *et al.*, 2008).

The parvicellular subparafascicular nucleus has been divided into medial and lateral subdivisions based on a dense population of galanin-immunoreactive fibers in the medial subdivision, and the presence of CGRP cells in the lateral subdivision (Coolen *et al.*, 2003a). In addition, the medial subdivision demonstrates *c-fos* expression following male ejaculation (Coolen *et al.*, 2004; Coolen *et al.*, 2003a) while the lateral subdivision does not (Coolen *et al.*, 2003a; D'Hanis *et al.*, 2007), providing functional evidence for the compartmentalization of the area (Coolen *et al.*, 2003a). Observations that TIP39 neurons are located predominantly medial to CGRP neurons (Brenner *et al.*, 2008) and that they express *c-fos* in association with ejaculation, as described below (Wang *et al.*, 2006a), suggests that parvicellular subparafascicular TIP39 neurons are located in the medial subdivision of the SPFp (Fig. 3). However, TIP39 and CGRP neurons extend further than the SPFp in the caudolateral direction while maintaining their medio-lateral separation suggesting that not only the SPFp but the whole PIL can be divided into medial and lateral parts, and that the TIP39 neurons are located in the medial (Fig. 3) while the 'CGRP nucleus' of the thalamus is in the lateral part of the PIL. Such division is also supported by the projection pattern of the TIP39 neurons in the PIL (discussed below), which is largely different from the striatal, amygdaloid, and perirhinal

cortical projections of the thalamic CGRP neurons (Dobolyi *et al.*, 2005; Inagaki *et al.*, 1990; Yasui *et al.*, 1991).

3.3 TIP39 cells in the medial paralemniscal nucleus (MPL)

There is a distinct group of TIP39-containing cell bodies in the rostral pons between bregma levels -8.0 to 8.5 mm (Dobolyi *et al.*, 2002; Faber *et al.*, 2007; John *et al.*, 2002), medial to the fibers of the lateral lemniscus, immediately dorsal to the rubrospinal tract and rostral to the Kölliker-Fuse nucleus (Fig. 4). When we initially mapped the distribution of TIP39 cell bodies in detail (Dobolyi *et al.*, 2003b), we tentatively named the area of TIP39 expression immediately medial to the auditory relay nuclei of the lateral lemniscus as the medial paralemniscal nucleus (MPL). However, the identification of this nucleus has not been straightforward without labeling TIP39 (Varga *et al.*, 2008). In the literature, often no distinction is made between oral reticular pontine and paralemniscal zones. Areas that include cells that correspond to the MPL have been referred to by a variety of anatomical names with poor topographical characterization, such as “lateral part of the nucleus reticularis pontis oralis” (Papez, 1926), “lateralmost nucleus reticularis pontis oralis” (Leichnetz *et al.*, 1978), “ventrolateral tegmental area” (Herbert *et al.*, 1997), or “dorsolateral pontomesencephalic reticular formation” (Haws *et al.*, 1989). The term “paralemniscal” has also been used without detailed topographical characterization when describing an area in the “paralemniscal zone” whose stimulation elicited pinna movement in cats (Henkel and Edwards, 1978), a group of neurons whose activity changed in response to noxious stimuli in the “paralemniscal reticular formation” (Hardy *et al.*, 1983), a group of neurons expressing *c-fos* in response to suckling in the “caudal portion of the paralemniscal nucleus” (Li *et al.*, 1999), a group of “audiovocal” neurons in the “paralemniscal tegmentum” (Metzner, 1993), a group of neurons whose stimulation elicited vocalization in the “paralemniscal tegmental area” in bats (Fenzl and Schuller, 2007; Schuller and Radtke-Schuller, 1990), and the “ventral paralemniscal area” in squirrel monkey (Hage and Jurgens, 2006; Hannig and Jurgens, 2006). Furthermore, the existence of a cell group probably corresponding to the MPL described in the present study was mentioned in early studies (Fuse, 1926; Wünscher *et al.*, 1965), and also more recently, as the “caudal part of the paralemniscal nucleus” (Andrezik and Beitz, 1985). However, the MPL is different from the paralemniscal nucleus described in a more rostral and lateral location (Olszewski and Baxter, 1982; Paxinos and Watson, 1998; Taber, 1961). The term “medial paralemniscal nucleus,” introduced originally as the location of TIP39 neurons (Dobolyi *et al.*, 2003b), has been adopted by recent editions of the widely used Paxinos rat brain atlas (Paxinos and Watson, 2005).

Cells of the MPL are distinguished from those in adjacent areas by their organization into dorsolaterally oriented cell columns separated by 20–50- μ m wide cell-free zones (Fig. 4C), probably occupied by fibers of the lateral lemniscus that pass through the region (Varga *et al.*, 2008). The ventral border of the MPL is the rubrospinal tract, which is easily distinguished and clearly separated by the abrupt end of the cell columns (Fig. 4B). However, lateral to the rubrospinal tract, the MPL extends somewhat ventrally, which gives the nucleus a triangular shape with ventral, dorsal, and medial angles (Dobolyi *et al.*, 2003b; Paxinos and Watson, 2005). In addition, in the rostral half of the MPL, a small group of large acetylcholinesterase-positive cells of the epirubrospinal nucleus (Paxinos and Butcher, 1985) is located between the medial part of the rubrospinal tract and the medial part of the MPL. The rostral part of the MPL is embedded between the pedunculopontine tegmental and the retrorubral nuclei, from which the MPL is separated by a zone of lower cell density (Fig. 4E). Medially, the MPL borders on the oral part of the pontine reticular formation and the pedunculopontine tegmental nucleus (Fig. 4B,E). The MPL narrows dorsally between the caudal part of the pedunculopontine tegmental nucleus and the dorsal nucleus of the lateral lemniscus, giving the nucleus a cone shape (Fig. 4B). The lateral border of the MPL is the intermediate nucleus of the lateral

lemniscus (Fig. 4B). The caudal borders of the MPL are the region of the A7 noradrenaline cell group medially and the Kölliker-Fuse nucleus laterally (Fig. 4E,F). At present, the functional relevance of the topographical relationship between the TIP39 and these noradrenaline neurons is not known.

The MPL is distinguished from adjacent brainstem nuclei both by its cytoarchitecture and by its afferent connections (Varga *et al.*, 2008). Morphologically, three cell populations can be distinguished within the MPL. About one-third of the cells are glial cells. There are about 300-600 TIP39 neurons per side in young adult rats and they account for about 75% of the neuronal population. Non-TIP39 neurons, constituting the third group, are somewhat larger than TIP39 neurons. The distributions of the three cell populations entirely overlap, and all of them participate in the formation of cell columns within the MPL. It is not clear whether the columnar cellular arrangement is an inherent property of the MPL or whether it results from the abundant fiber bundles passing through the MPL (Varga *et al.*, 2008).

3.4 Functional implications derived from the location of TIP39 neurons

The brain areas that contain TIP39 neurons are not very well characterized functionally. However, the literature does suggest some functions, which based on their positions, TIP39 neurons might be involved in. Thus, we briefly outline the most important relevant functions.

3.4.1 Functional associations of the PVG—The PVG is a site of stimulation-induced analgesia (Peschanski and Mantyh, 1983; Rhodes and Liebeskind, 1978). Potent analgesia is obtained in rats following electrical stimulation in the gray matter surrounding the caudal portion of the third ventricle and the midline area of the caudal thalamus that is comparable to that produced by stimulation of the caudal periaqueductal gray. Analgesia outlasts the period of brain stimulation, and is not due to a generalized motor debilitation of the animal (Rhodes and Liebeskind, 1978). In addition, some neurons in the area are activated by noxious stimuli (Dong *et al.*, 1978; Sugiyama *et al.*, 1992). Cold exposure (10 °C), that does not necessarily cause a change in abdominal temperature, induced *c-fos* in some PVG neurons (Baffi and Palkovits, 2000; Kiyohara *et al.*, 1995; Miyata *et al.*, 1995). In contrast, warm ambient temperature (33 °C) did not induce *c-fos* expression in the PVG (Kiyohara *et al.*, 1995). It has also been reported that the *c-fos* expression in the PVG significantly outlasts the cold exposure (Miyata *et al.*, 1995), suggesting that it may have a role in the maintenance of homeostasis during adaptation to cold stress (Baffi and Palkovits, 2000).

3.4.2 Functional associations of the PIL—Studies using electrical stimulation (Shimura and Shimokochi, 1991), lesions (Maillard and Edwards, 1991), and mapping immediate early gene activation patterns (Coolen *et al.*, 1997), implicate the area corresponding to the medial part of the PIL in sexual function. Reportedly, *c-fos* is expressed in the area following ejaculation, and earlier in the context of mating (Coolen *et al.*, 1997, 1998; Sachs and Meisel, 1988; Veening and Coolen, 1998). Mating-activated neurons project to other regions that show *c-fos* expression with ejaculation, including the posterodorsal preoptic nucleus, the lateral part of the posterodorsal medial amygdala, and the medial cell group of the sexually dimorphic preoptic area (Coolen *et al.*, 1997; Heeb and Yahr, 2001; Sachs and Meisel, 1988; Veening and Coolen, 1998). In rat, *c-fos* was induced in some TIP39 neurons following mating behavior suggesting that these cells may be involved in male sexual function (Wang *et al.*, 2006a). Significant *c-fos* activation in TIP39 neurons took place in the SPFP as well as more lateral parts of the PIL. The number of dual labeled cells and the percentages of TIP39 cells in the PIL that express Fos, as well as the percentages of Fos-positive cells that co-expressed TIP39 significantly increased following one or two ejaculations, compared to animals that displayed only intromissions. Furthermore, males with two ejaculations had higher numbers of dual labeled cells compared to males with one ejaculation.

Based on its connections the posterior intralaminar complex of the thalamus was suggested to process auditory inputs to emotional brain centers, including the amygdala (LeDoux *et al.*, 1990; Linke and Schwegler, 2000; Namura *et al.*, 1997). Indeed, the area ventromedial to the medial geniculate body, which includes the posterior intralaminar thalamic nucleus, contains neurons in which *c-fos* is induced following high-intensity auditory stimuli (Burow *et al.*, 2005; Campeau and Watson, 1997). Exposure to 30-min of 105 dB white noise induces *c-fos* in a large portion of the TIP39 neurons in the PIL, but not in the PVG TIP39 neurons (Palkovits *et al.*, 2004, 2009a). Since the hypothalamic paraventricular nucleus receives projections from the PIL (Campeau and Watson, 2000; Palkovits *et al.*, 2004) these findings suggest that TIP39 neurons in the PIL may be involved in mediation of audiogenic stress signals that reach the hypothalamus, and signals that contribute to acoustic fear conditioning that involve the amygdala (Palkovits *et al.*, 2004).

3.4.3 Functional associations of the MPL—As part of the dorsolateral pontomesencephalic tegmentum, the MPL is implicated in brainstem pain-regulatory systems. Stimulation of this region elicits analgesia (Basbaum *et al.*, 1977; Haws *et al.*, 1989) and inhibits the response of spinothalamic tract cells to noxious stimuli (Girardot *et al.*, 1987), and these effects are probably unrelated to the A7 noradrenergic cells (Zhao and Duggan, 1988). Furthermore, the firing rate of some paralemniscal neurons changes in response to noxious stimuli (Hardy *et al.*, 1983).

The paralemniscal vocalization center of bats (Metzner, 1996) and squirrel monkeys (Hage and Jurgens, 2006; Hannig and Jurgens, 2006) occupies a position similar to the paralemniscal TIP39 neurons, medial to the intermediate nucleus of the lateral lemniscus. A group of “audio-vocal neurons” in this area responds to some auditory stimuli and their stimulation evokes vocalization (Fenzl and Schuller, 2007; Hage and Jurgens, 2006; Metzner, 1993; Schuller and Radtke-Schuller, 1990). In cats, stimulation of an area with a description similar to the MPL elicits pinna movement (Henkel and Edwards, 1978), its connections suggests that it conveys information from the superior colliculus to the motor facial nucleus (Henkel, 1981).

Another function in which MPL TIP39 neurons might be involved is the influence of pup exposure and suckling on lactation. Pup exposure and suckling induce *c-fos* expression in the bed nucleus of the stria terminalis, medial amygdala, lateral parabrachial nucleus, caudal part of the periaqueductal gray, and “caudal part of the paralemniscal nucleus immediately dorsolateral to the A7 cell group” (Li *et al.*, 1999). This pattern would be consistent with activated TIP39 neurons activating other cells in their termination fields following a pup exposure and suckling stimulus.

3.5 Development of TIP39 neurons

There is good agreement on the pattern of TIP39 expression evaluated using *in situ* hybridization and quantitative RT-PCR for TIP39 mRNA and immunolabeling of TIP39 peptide during embryonic and postnatal development in rat (Brenner *et al.*, 2008; Dobolyi *et al.*, 2006b). As soon as TIP39 labeled neurons appear during development, their localization is similar to that in adult except for the amygdala, where TIP39 containing neurons appear transiently during embryonic development as described below.

In the posterior thalamus, there is a dramatic difference between the developmental expression of TIP39 by medially located periventricular and the laterally located posterior intralaminar neurons. TIP39 appears in and around the posterior intralaminar nucleus at embryonic day (ED)-14.5. The number of TIP39-ir neurons increases markedly by ED-16.5. At this age, TIP39-ir neurons occupy a large area in and around the posterior intralaminar thalamic nucleus including a few cells rostromedially in the parvocellular subparafascicular nucleus. Between ED-16.5 and postnatal day (PND)-5, a gradual decrease in the number of posterior intralaminar

TIP39 neurons occurs, and the number of TIP39 neurons in the area remains low after that time point. The distribution of TIP39 neurons and the intensity of their immunolabeling are the same in male and female rats. TIP39 neurons in the posterior intralaminar thalamic nucleus elaborate TIP39-ir fibers by ED-18.5, which can be followed rostrally in the supraoptic decussations towards the hypothalamus. In contrast, TIP39 neurons in the PVG are first visible only on the first postnatal day (Brenner *et al.*, 2008). In this region, the intensity of labeling as well as the number of TIP39 neurons increase substantially thereafter until PND-14. After that, TIP39 levels gradually decrease in the PVG, are markedly reduced by PND-125, and remain very low thereafter (Dobolyi *et al.*, 2006b). The disappearance of TIP39 from cell bodies and fibers only means that TIP39 is not detectable in them, and provides no information regarding whether TIP39 neurons degenerate during development or whether their TIP39 expression decreases. At present, there are no independent markers for TIP39 neurons that would allow testing of these possibilities.

In the medial paralemniscal nucleus, TIP39-ir neurons first appear at ED-14.5 and the number of TIP39-ir neurons as well as the intensity of their labeling increases thereafter (Brenner *et al.*, 2008). At PND-5, TIP39-ir fibers can be observed in the medial paralemniscal nucleus as they leave TIP39-ir cell bodies and project dorsally and ventrally. In contrast to the markedly different embryonic development, the postnatal peak and decrease in the intensity of TIP39 labeling in the MPL is similar to that in the PVG described above (Dobolyi *et al.*, 2006b).

A group of TIP39 neurons appears in the amygdala at ED-16.5 (Brenner *et al.*, 2008). About 100-200 neurons are located in the anterolateral subdivision of the amygdalo-hippocampal transitional zone dorsal to the posterior part of the cortical amygdaloid nucleus and lateral to the posterior part of the medial amygdaloid nucleus, with some cells located in the adjacent posterior subdivision of the basomedial amygdaloid nucleus. The intensity of TIP39 immunolabeling decreases after ED-16.5. By PND-1, TIP39 mRNA expression is low and only faintly immunolabeled TIP39 neurons are visible in the amygdalo-hippocampal transitional zone in both sexes and these neurons are not observed at any later stages of postnatal development, although this area establish moderate density of TIP39-ir fibers during adulthood (Dobolyi *et al.*, 2003b). The posterior part of the amygdala is a relatively little studied brain region. Based on the expression of steroid receptors and its neuronal connections (Canteras *et al.*, 1992), the amygdalo-hippocampal transitional zone has been suggested to play a role in conveying hormonal information toward reproductive brain centers (Simerly, 2002).

3.6 Sexual dimorphism of adult TIP39 expression

In situ hybridization histochemistry and immunocytochemistry reveal the same distribution of TIP39-expressing perikarya in male and female rats and mice (Dobolyi *et al.*, 2006b; Faber *et al.*, 2007) and there is no sex difference in TIP39 mRNA expression in young animals. However, as the level of TIP39 mRNA and peptide decreases with age, it becomes sexually dimorphic in the posterior thalamus and medial paralemniscal nucleus. TIP39 expression is significantly higher in aged female than in aged male rats but even the female levels are markedly decreased as compared to younger animals (Dobolyi *et al.*, 2006b). This sex difference is not due to a particular estrus cycle stage of the female rats. Furthermore, 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 (Dobolyi *et al.*, 2006b). In turn, gonadectomy performed before PND-24 partially reversed the decrease in TIP39 levels, suggesting that the decrease is related to sexual steroid hormonal effects occurring during the period of sexual maturation (Lamming, 1994). The sex difference in the depletion of TIP39 expression, on the other hand, was still present in gonadectomized animals, suggesting it is set up by events prior to PND-24.

4 Neuronal input to TIP39 neurons

Brain regions with projections to TIP39 neuron containing regions are shown in Fig. 5. These areas were identified following retrograde tracer injection into the regions where TIP39 neurons are concentrated. Double labeling of TIP39 and the anterograde tracer injected into areas that project toward TIP39 neurons, which allows identification of inputs that make close appositions with TIP39 neurons, has been performed so far for only two regions that project to the MPL, as described below.

4.1 Neuronal input to TIP39 neurons in the PVG

Because of the elongated shape of the PVG, the inputs towards its rostral, middle and caudal parts have been investigated separately. A large number of brain regions provide significant input to the PVG (Fig. 5), and the projections to the different parts of the PVG are essentially the same (Wang *et al.*, 2006b). Furthermore, the majority of the afferent connections are ipsilateral, with only a small number of retrogradely labeled cells present contralaterally following tracer injections into the PVG (Wang *et al.*, 2006b). The forebrain regions that send significant projections to the PVG are the medial prefrontal, insular, and ectohippocampal cortices, the subiculum, the anterior and medial amygdaloid nuclei, and the lateral septum. The rostral portion of the intermediate part of the lateral septal nucleus (also called the lateral septal nucleus, pars anterior) displayed the highest density of retrogradely labeled cells following tracer injections into the PVG. The majority of the projections to the PVG from the diencephalon arise from hypothalamic regions, including the ventromedial nucleus, which contains a very high density of retrogradely labeled cells, the preoptic area, the anteroventral periventricular nucleus, the lateral hypothalamic area, the anterior, posterior and dorsomedial hypothalamic, and the dorsal premammillary nuclei. Other diencephalic regions that project to the PVG include the zona incerta and the area ventromedial to the medial geniculate body where TIP39 expressing neurons of the PIL are located. In the brainstem, the periaqueductal gray projects to the PVG with its dorsolateral division containing a particularly high density of labeled cells throughout its rostro-caudal extent. In addition, deep layers of the superior colliculus, cortical areas of the inferior colliculus, the cuneiform nucleus, the medial paralemniscal nucleus, and the parabrachial nuclei also send significant projection to the PVG (Wang *et al.*, 2006b). The afferent connections of the PVG are consistent with previous anterograde studies on projections to the area from auditory cortices (Arnault and Roger, 1990; Yasui *et al.*, 1990), the anterior hypothalamic nucleus (Risold *et al.*, 1994), the dorsal premammillary nucleus (Canteras *et al.*, 1992), the zona incerta (Watanabe and Kawana, 1982), the area medial to the medial geniculate body (LeDoux *et al.*, 1985), and the inferior colliculus (Arnault and Roger, 1990; Kudo *et al.*, 1984; Yasui *et al.*, 1990). The afferent connections of the PVG are markedly different from those of the midline and intralaminar thalamic nuclei including the parafascicular nucleus (Van der Werf *et al.*, 2002). The extensive hypothalamic but limited thalamic and brainstem inputs imply that the PVG does not belong to this group of thalamic nuclei, as it has been suggested by previous reports (LeDoux *et al.*, 1985; Rub *et al.*, 2002). The PVG has a number of common afferent connections with the periaqueductal gray including insular, ectohippocampal, and perirhinal cortical areas, the hypothalamus, the zona incerta, the superior and inferior colliculi, the nucleus cuneiformis, and the parabrachial nuclei (Beitz, 1982; Shipley *et al.*, 1991).

4.2 Neuronal input to TIP39 neurons in the PIL

Although there are no data on the neuronal inputs of the posterior intralaminar thalamic nucleus, the afferent connections of the other part of the PIL, the SPFP have been described (Coolen *et al.*, 2003b). Neurons in the medial prefrontal, insular and somatosensory cortex, the substantia innominata, the medial and lateral preoptic area, the hypothalamic paraventricular nucleus, the anterior amygdaloid area, the medial nucleus of the amygdala, the ventral tegmental area, the

parabrachial nuclei, the locus coeruleus, the nucleus of the solitary tract, and the spinal trigeminal nucleus all project predominantly to the medial subdivision of the SPFP while the lateral subdivision receives specific inputs from auditory and visual brain regions (Coolen *et al.*, 2003b). In addition, both subdivisions receive significant projections from the auditory cortex, the lateral septal nucleus, the anterior and hypothalamic ventromedial nuclei, the zona incerta, the periaqueductal gray, the deep layers of the superior colliculus, the cortical layers of the inferior colliculus, the cuneiform nucleus, and the pedunculopontine tegmental nucleus (Coolen *et al.*, 2003b). The afferent connections of the medial subdivision of the SPFP are very similar to those of the PVG (Fig. 5). However, the medial subdivision of the SPFP but not the PVG receives dense projections from the somatosensory cortex, the hypothalamic paraventricular nucleus, the ventral tegmental area, the locus coeruleus, the nucleus of the solitary tract, and the spinal trigeminal nucleus. As described above, *c-fos* expression is induced in TIP39 neurons of the PIL in male rats following ejaculation but much less so following intromission without ejaculation. It suggests that these TIP39 neurons are part of the afferent circuits that process genital-somatosensory information related to ejaculation that contribute to mating and mating-induced changes in reproductive behaviors. The visceral, somatosensory, proprioceptive, and noxious information that can reach the medial subdivision of the SPFP (Coolen *et al.*, 2003b) support the proposal that TIP39 neurons in the area are in position to integrate and transmit information related to copulation (Wang *et al.*, 2006a). Finally, TIP39 neurons in the PIL can also be activated by high-intensity auditory input (Palkovits *et al.*, 2004, 2009a), although the precise pathway for auditory information has not yet been defined.

4.3 Neuronal input to TIP39 neurons in the MPL

The MPL has afferent neuronal connections distinct from adjacent brain regions including major inputs from the auditory cortex (Fig. 6B), the medial part of the medial geniculate body, superior colliculus, external and dorsal cortices of the inferior colliculus, periolivary area, lateral preoptic area, hypothalamic ventromedial nucleus, lateral and dorsal hypothalamic areas, subparafascicular and posterior intralaminar thalamic nuclei, periaqueductal gray, and the cuneiform nucleus (Varga *et al.*, 2008) (Fig. 5). In addition, injection of anterograde tracer into the auditory cortex and the hypothalamic ventromedial nucleus confirmed distinct projections from these areas to the MPL vs. the adjacent brainstem (Fig. 6C,D), which is in agreement with previous literature on projections of the hypothalamic ventromedial nucleus (Canteras *et al.*, 1994) and the auditory cortex (Perales *et al.*, 2006), respectively. Furthermore, the projections from these two regions appear to terminate on TIP39 neurons based on confocal microscopy of tracer/TIP39 double labeling (Varga *et al.*, 2008).

Like the external cortex of the inferior colliculus and the lateral subdivision of the thalamic PIL described above, the MPL receives numerous projections from the auditory cortex (Herbert *et al.*, 1991; Varga *et al.*, 2008; Winer *et al.*, 1998), demonstrates *c-fos* activation following loud noise (Campeau and Watson, 1997; Palkovits *et al.*, 2004), and does not receive significant direct projections from lower brainstem auditory nuclei (Faye-Lund and Osen, 1985; Saldaña and Merchán, 2005; Varga *et al.*, 2008). The massive collicular and cortical inputs have been demonstrated to contribute to the induction of *c-fos* in the external cortex of the inferior colliculus and PIL in response to high-intensity auditory stimuli (Burow *et al.*, 2005; Sun *et al.*, 2007). Therefore, collicular and cortical inputs are potentially the anatomical substrate for evoking *c-fos* activation in TIP39 neurons of the MPL, too (Palkovits *et al.*, 2004). The strong interconnections of the MPL, the PIL and the external cortex of the inferior colliculus as well as their common non-tonotopic organization (Clopton *et al.*, 1974) suggest that they may be involved in related neural functions. Potential functions include the integration of auditory and somatosensory information (Ledoux *et al.*, 1987; Zhou and Shore, 2006), the modulation of vigilance and attention by auditory stimuli (Jane *et al.*, 1965), mediating cortical contributions to filtering (Bregman, 1990) information such as the global location of a sound in space

(Middlebrooks *et al.*, 1994), regulating orientation behaviors, e.g. head and pinna movement and audio-vocal interfaces (Thompson, 1997), providing information to a map of auditory space (King *et al.*, 1998), and to the coordination of responses to sound (Schuller *et al.*, 1991), detecting novelty (Perez-Gonzalez *et al.*, 2005) modulating the acoustic startle response (Yeomans *et al.*, 2002), and processing auditory inputs to emotional brain centers e.g. for acoustic fear conditioning (LeDoux *et al.*, 1990).

5 Projections of TIP39 neurons

Widespread TIP39 containing fibers originate from one of the three discrete groups of TIP39 neurons described above, in the PVG, PIL or MPL. Different techniques have been used to establish the projection patterns of individual groups of TIP39 neurons. First, the 3 brain regions containing TIP39 neurons were lesioned and disappearance of TIP39-containing fibers analyzed (Dobolyi *et al.*, 2003a). A combination of targeted bilateral and unilateral lesions provided a great deal of information on the projection of individual TIP39 cell groups (Dobolyi *et al.*, 2003a). Furthermore, anterograde tracers were injected into brain regions containing TIP39 cell bodies, particularly into the PVG (Wang *et al.*, 2006c) and the PIL (Campeau and Watson, 2000). In addition, injections of retrograde tracers into brain regions containing TIP39 fiber terminals can provide information on the origin of projections even if more than one TIP39 cell groups project to the area or if the projections are bilateral. Unfortunately, only a limited amount of work has been performed with this method (Wang *et al.*, 2006c).

5.1 Distribution of TIP39-containing fibers and fiber terminals

TIP39-immunoreactive fibers have been mapped in and carefully compared between the rat and mouse brain (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007). The only difference observed was in the medial preoptic area, where the intensity of TIP39 fibers was somewhat higher in mice, so the following discussion is applicable to both species. The topographical distribution of TIP39-containing fibers is unique; there is no other neuropeptide or marker known to have a similar distribution. Brain regions containing high densities of TIP39 fibers can be grouped into auditory regions, endocrine/hypothalamic regions, limbic regions, and viscerosensory/nociceptive regions. Auditory regions where TIP39 fibers are abundant include the periolivary area, the medial nucleus of the trapezoid body, the external cortex of the inferior colliculus, deep layers of the superior colliculus, the medial nucleus of the medial geniculate body, and the area ventral to it, and the ectorhinal cortex. Endocrine/hypothalamic brain areas that have a high density of TIP39 fibers are the medial preoptic area, the hypothalamic paraventricular, periventricular, and dorsomedial nuclei (Fig. 7), the arcuate nucleus (Fig. 7), the lateral and dorsal hypothalamic areas (Fig. 7), and the premamillary nuclei. Additional limbic structures that contain a high density of TIP39 fibers include the medial prefrontal cortex (particularly the infralimbic cortex), the shell and cone portions of the nucleus accumbens, the lateral septal nucleus, the bed nucleus of the stria terminalis, the thalamic paraventricular nucleus (Fig. 7), the fundus striati (or amygdalo-striatal transitional zone), the ventral subiculum, and the central and medial amygdaloid nuclei (Fig. 7). Finally, TIP39 fibers are also abundant in some additional brain regions, which can loosely be considered viscerosensory/nociceptive including the lateral parabrachial nucleus, the anterior pretectal nucleus, and the periaqueductal gray. In addition, TIP39 fibers are also present in the dorsolateral funiculus of the spinal cord. Classification of the brain regions containing TIP39 fibers is of course arbitrary because most brain regions participate in multiple functions. In particular, the periaqueductal gray and the premamillary nuclei could easily be considered limbic structures, the central amygdaloid nucleus as viscerosensory, the thalamic paraventricular nucleus as nociceptive, and so on. Despite the obvious problems of grouping brain structures containing TIP39 fibers, it may help with the consideration of functions modulated by TIP39.

5.2 Projections of PVG TIP39 neurons

Following electrolytic lesions of the PVG, the density of TIP39 fibers decreased in the medial prefrontal cortex, the shell and cone portions of the nucleus accumbens, the lateral septum (Fig. 8A), the bed nucleus of the stria terminalis, the amygdaloid nuclei, the fundus striati, the ventral subiculum, the thalamic paraventricular nucleus, the hypothalamus, and the periaqueductal gray, suggesting that these regions receive projections from TIP39 neurons in the PVG (Dobolyi *et al.*, 2003a). Unilateral lesions demonstrated that the projections are dominantly ipsilateral (Dobolyi *et al.*, 2003a). Projections to these brain regions from the PVG were supported by anterograde tracer injection (biotinylated dextran amine; BDA) into the PVG (Wang *et al.*, 2006c). Part of the trajectory of anterogradely labeled fibers could be followed from the PVG. A group of fibers leaves the area dorsolaterally, then after a hook-shaped turn they enter the epithalamus and run rostral-wards in the paraventricular nucleus of the thalamus. Some other fibers run rostrally from the nucleus, just dorsal to the medial lemniscus and terminate, most probably in thalamic nuclei. Another group, which comprises the most substantial number of fibers, joins the zona incerta and the supraoptic decussations and enters the basal forebrain/substantia innominata area. From here, the fibers divide into branches to limbic cortical, septal and amygdaloid areas (Wang *et al.*, 2006c). The patterns of anterogradely labeled fibers following injections into rostral as well as caudal part of the PVG were similar to the distribution of TIP39-ir fibers in many areas of the brain, including the medial prefrontal and entorhinal cortices, the shell part of the accumbens nucleus, the lateral septal nucleus, the bed nucleus of the stria terminalis, the central amygdaloid nucleus, the fundus striati, the paraventricular and posterior intralaminar nuclei of the thalamus, the medial preoptic area, the posterior hypothalamic nucleus, the zona incerta, and the periaqueductal gray (Wang *et al.*, 2006c). Furthermore, these regions all contained fibers double-labeled with BDA and TIP39 supporting the projection of TIP39 fibers to these regions from the PVG. Retrograde tracer injections into the lateral septal nucleus and the fundus striati resulted in labeled TIP39 perikarya in the PVG but not in other regions containing TIP39 neurons (Wang *et al.*, 2006c) suggesting that the PVG provides the only TIP39-containing input to these two brain regions. The percentage of retrogradely labeled TIP39 neurons in the PVG was high indicating that TIP39 neurons are a major PVG output neuron. Double labeled neurons were evenly distributed in the PVG suggesting that TIP39 neurons in the PVG constitute a single cell population (Wang *et al.*, 2006c). In contrast, some of the hypothalamic areas including the anterior, paraventricular, arcuate and dorsomedial nuclei did not contain fibers double-labeled with TIP39 and BDA following PVG BDA injection. Most of these nuclei actually contained a higher density of TIP39-ir than BDA labeled fibers (Wang *et al.*, 2006c). These data suggest that the major source of TIP39 fibers in most hypothalamic areas is not the PVG.

5.3 Projections of PIL TIP39 neurons

Following lesions of the area medial and ventromedial to the medial geniculate body, including the PIL, TIP39 fibers almost completely disappeared from the ipsilateral amygdala. A sharp reduction was observed in the density of TIP39 fibers in the ipsilateral hypothalamus particularly in the paraventricular (Fig. 8B) and dorsomedial nuclei. In addition, smaller, but visible reductions in the density of TIP39 fibers were observed ipsilateral to the lesion in many other forebrain regions including the medial prefrontal cortex, the nucleus accumbens, and the bed nucleus of the stria terminalis (Dobolyi *et al.*, 2003a). Another study injecting anterograde tracer in an area likely to correspond to the region expressing TIP39 in the PIL described widespread neuronal projections in the rat brain (Campeau and Watson, 2000). The results confirmed previous retrograde studies on projections to the amygdaloid complex (Heeb and Yahr, 2001; LeDoux *et al.*, 1985; Ottersen and Ben-Ari, 1979; Turner and Herkenham, 1991; Yasui *et al.*, 1991), temporal cortex (LeDoux *et al.*, 1985; Turner and Herkenham, 1991), preoptic area (Coolen *et al.*, 1998; Heeb and Yahr, 2001; Simerly and Swanson, 1986), and the ventromedial nucleus of the hypothalamus (LeDoux *et al.*, 1985) and extended these

findings by showing fibers and putative terminals in several additional areas, including the lateral ventral and intermediate septal nuclei, several subdivisions of the bed nucleus of the stria terminalis and substantia innominata, and many hypothalamic structures including the preoptic area, the retrochiasmatic area, the paraventricular nucleus, the anterior, lateral, and dorsal hypothalamic areas, the dorsomedial nucleus, the tuber cinereum area (Campeau and Watson, 2000). In addition, retrograde studies confirmed projection from the PIL to the hypothalamic paraventricular nucleus (Campeau and Watson, 2000; Palkovits *et al.*, 2004). Descending fibers from the SPFP have also been reported, including projections to the PVG, the periaqueductal gray, the external cortex of the inferior colliculus, and the superior olivary complex (Yasui *et al.*, 1992). Comparing the studies using neuronal tracers and lesion, it is clear that TIP39 neurons in the PIL project to the amygdala and hypothalamus. They may also provide fibers to the bed nucleus of the stria terminalis and substantia innominata. Furthermore, TIP39 neurons in the PIL may also contribute to TIP39 fibers in the ectorhinal cortex, the periaqueductal gray, the external cortex of the inferior colliculus, and the periolivary area.

5.4 Projections of MPL TIP39 neurons

Following lesion of the MPL, there was an almost complete disappearance of TIP39 fibers from the deep layers of the superior colliculus, the external cortex of the inferior colliculus (Fig. 8C), the cuneiform nucleus, the lateral parabrachial nucleus, the medial nucleus of the trapezoid body, the periolivary area, as well as from the spinal cord, while a moderate density of TIP39-containing fibers remained in the periaqueductal gray (Dobolyi *et al.*, 2003a). Unfortunately, anterograde injections into an area that can be reliably identified as the location of TIP39 neurons in the MPL have not been described. Consequently, the data are scarce regarding the topography of the projection of fibers from the MPL.

5.5 Interconnection of brain regions containing TIP39 neurons

Injection of retrograde tracer into the PVG revealed that cells from the PIL and the MPL, the other two brain regions that contain TIP39 neurons, project to the PVG (Wang *et al.*, 2006b). Although the distribution of retrogradely labeled and TIP39 neurons largely overlapped, no double-labeled cells were observed, suggesting that non-TIP39 neurons project to the other TIP39 expressing brain regions (Wang *et al.*, 2006b). Similarly, following injection of retrograde tracer into the MPL, the PVG and the PIL contained retrogradely labeled cells. The distribution of retrogradely labeled cells in both regions of the posterior thalamus was similar to that of TIP39 neurons in the area. However, cells double-labeled with the retrograde tracer and TIP39 were not observed, suggesting that non-TIP39 PVG and PIL neurons project to the MPL (Varga *et al.*, 2008). In addition, following MPL injection a high density of retrogradely labeled cells was present in the contralateral MPL. These retrogradely labeled cells were also TIP39 negative (Varga *et al.*, 2008). Apart from the mutual interconnection of the TIP39-expressing brain regions by non-TIP39 projections, another strong similarity is the massive input to all three areas from the hypothalamic ventromedial nucleus as well as from the significant inputs from the preoptic area, the periaqueductal gray, the deep layers of the superior colliculus, and the cortical layers of the inferior colliculus (Coolen *et al.*, 2003b; Varga *et al.*, 2008; Wang *et al.*, 2006b). These findings suggest that TIP39 neurons in the three brain regions might participate in some common functions despite their apparently different projections.

5.6 Summary of the origin of TIP39 fibers in different brain regions

The major projections of the three groups of TIP39 neurons are summarized in Figure 9. Brain regions that receive TIP39 fibers predominantly from the PVG include the medial prefrontal cortex, the lateral septal nucleus, the bed nucleus of the stria terminalis, the thalamic paraventricular nucleus, the ventral subiculum, and the fundus striati. Brain regions that receive most of their TIP39 fibers from the PIL are the hypothalamus and the amygdala. TIP39-ir fibers

that reach the hypothalamic nuclei from the PIL travel mainly through the supraoptic decussations and the zona incerta, but neurons in the PVG may also project *via* these two pathways (Palkovits *et al.*, 2009b). Available evidence suggests that all three TIP39-expressing brain regions project to the arcuate nucleus, and that hypothalamic and amygdaloid regions receive a significant number of TIP39 fibers from the PIL as well as the PVG. The MPL provides most TIP39 fibers to brainstem regions including the deep layers of the superior colliculus, the external cortex of the inferior colliculus, the lateral parabrachial nucleus, the periolivary area, the medial nucleus of the trapezoid body, and the spinal cord. However, the external cortex of the inferior colliculus and the spinal cord receive projections from the PVG as well as the PIL, and some of these projections could be TIP39 fibers. Similarly, the superior periolivary area may also receive TIP39 fibers from the PIL. The periaqueductal gray receives a comparable amount of TIP39 fibers from the PVG and MPL, with a smaller potential input from the PIL.

6 The PTH2R as the receptor for TIP39

Since previous reviews have summarized the identification of the PTH2R and compared it to the PTH1R (Hoare and Usdin, 2001; Usdin, 2000; Usdin *et al.*, 2002; Usdin *et al.*, 2000), in the present review, we only briefly describe the pharmacology of PTH receptors with an emphasis on the PTH2R, and focus on recent developments in the field including PTH receptors in zebrafish, signal transduction of PTH receptors, structure-activity studies involving TIP39, and the recent development of antagonists of the PTH2R.

6.1 PTH receptors

The PTH1R was identified by expression cloning in 1991 (Juppner *et al.*, 1991). It is activated with equal potency by PTH and PTHrP and is also referred to as the PTH/PTHrP receptor. The parathyroid hormone 2 receptor (PTH2R) was identified on the basis of its sequence homology with other polypeptide-recognizing receptors following a screen that used PCR with degenerate primers (Usdin *et al.*, 1995). The PTH receptors are seven transmembrane domain receptors belonging to the type II (or family B) class of G protein-coupled receptors (Usdin *et al.*, 2002), membership in which is identified on the basis of sequence similarity, particularly within the transmembrane domains (Harmar, 2001). PTH receptors show the highest degree of sequence similarity to other hormone receptors belonging to the B1 receptor subfamily, which includes receptors for calcitonin and related peptides, corticotropin releasing hormone and related peptides, gastric inhibitory polypeptide, glucagon and related peptides, growth hormone releasing hormone, parathyroid hormone and related peptide, pituitary adenylate cyclase activating peptide, secretin, and vasoactive intestinal peptides. In addition, the PTH receptors have other features that are characteristic of the B1 receptor subfamily including a potential signal sequence and a hydrophilic extracellular domain in the amino terminus that contributes significantly to ligand binding (Harmar, 2001; Usdin *et al.*, 1995). The overall amino acid sequence similarity of the PTH1R and PTH2R is 70% and they share at least 30% amino acid sequence similarity to other hormone receptors belonging to the B1 receptor subfamily (Usdin *et al.*, 1995).

Homologs of the mammalian PTH1R and PTH2R as well as a third PTH activated receptor have been identified in zebrafish (Rubin *et al.*, 1999; Rubin and Juppner, 1999). The zebrafish PTH1R is pharmacologically similar to the mammalian PTH1Rs. Rat PTH and PTHrP activate it with very similar potency (Rubin *et al.*, 1999). The zebrafish PTH2R is much like the rat PTH2R (Rubin *et al.*, 1999) as described below. In contrast, the zebrafish PTH3R appears to be somewhat similar to the PTH1R based on its binding affinities and potent activation by rat PTHrP and PTH (Rubin and Juppner, 1999). However, the potency of human PTH is considerably less than that of rat PTH or PTHrP for activation of the zebrafish PTH3R (Hoare *et al.*, 2000b). Thus, the ligand specificity of the zebrafish PTH3R does not clearly match any

of PTH receptors that have been pharmacologically characterized in mammals and its signal transduction pathway differs from that of the zebrafish PTH1R (Rubin and Juppner, 1999) as described below. Endogenous ligands for the zebrafish PTH-like receptors have been reported. TIP39 homologues in fish (Papasani *et al.*, 2004; Shoemaker *et al.*, 2006) were described above. In addition, a PTHrP homologue has been identified in fugu fish, and this peptide potently activates the zebrafish PTH1 and PTH3Rs (Rubin and Juppner, 1999) suggesting that a zebrafish PTHrP could normally act on these receptors. Two additional natural ligands, zPTH1 and zPTH2, were found by searching the zebrafish genomic database (Gensure *et al.*, 2004). These genes are also present in the Japanese pufferfish genome (Gensure *et al.*, 2004). Fish PTH1 and PTH2 have significant sequence homologies and similar genomic structures to mammalian PTHs (Gensure *et al.*, 2004). In addition, they activate the human and zebrafish PTH1R. zPTH2(1–34) is, however, approximately 30-fold less potent at the zPTH1R than hPTH(1–34), hPTHrP(1–36), and zPTH1(1–34). When tested with zPTH3R, zPTH1(1–34) and hPTHrP(1–36) showed similar potencies, whereas the potency of zPTH2(1–34) was moderately reduced (Gensure *et al.*, 2004). Overall, the PTH system appears more complex in fish than in mammals. However, fish do not have a parathyroid gland, and the functions of PTH and related polypeptides in fish remain to be elucidated (Gensure and Juppner, 2005).

6.2 Signal transduction pathways of the PTH receptors

One interesting functional characteristic of this family of G-protein coupled receptors is their potential to activate more than one second messenger cascade. Dual second messenger signaling has also been demonstrated for a number of receptors that are not within the secretin receptor family (Zhu *et al.*, 1994), but the evidence that dual signaling occurs in native tissues is particularly strong for secretin family receptors and especially for the PTH1R (Chorev, 2002; Gensure *et al.*, 2005). The activated PTH1R initiates a cascade of intracellular processes primarily by signaling through the α -subunit of the stimulatory G-protein, Gs (Schwindinger *et al.*, 1998) which in turn leads to cAMP synthesis and the activation of protein kinase A (Segre *et al.*, 1992). The PTH1R can also signal through Gq, thereby activating phospholipase C (Friedman *et al.*, 1999), increasing intracellular inositol trisphosphate and intracellular calcium ion levels (Tanaka *et al.*, 1995). In addition, PTH1R signaling through the mitogen-activated protein kinase pathway has been shown in several tissues (Chan *et al.*, 2001; Miao *et al.*, 2001). In fact, stimulation of the PTH1R may lead to the activation of the mitogen-activated protein kinase pathway by G protein-independent beta-arrestin signaling as well (Gesty-Palmer *et al.*, 2006). The zebrafish PTH1R also activates multiple signal transduction pathways. However, a potentially significant difference between the zebrafish PTH1R and PTH3R is that activation of the PTH1R stimulates inositol phosphate release in addition to cAMP formation, while the PTH3R does not appear to be coupled to stimulation of phospholipase C (Rubin and Juppner, 1999).

Signal transduction *via* the PTH2R has also been studied, although in less detail. Activation of the PTH2R in stably transfected HEK-293 cell lines (Behar *et al.*, 1996), and in COS-7 cells (Della Penna *et al.*, 2003; Hoare *et al.*, 1999; Hoare *et al.*, 2000a; Usdin *et al.*, 1995; Usdin *et al.*, 1999b), results in the accumulation of cAMP. In addition, PTH also increases (Behar *et al.*)_i in HEK293 cells (Behar *et al.*, 1996; Della Penna *et al.*, 2003) and in COS-7 cells (Goold *et al.*, 2001) transfected with the hPTH2R. The TIP39 stimulated increase in $[Ca^{2+}]_i$ is typified by an initial rapid onset with a peak at about 20 s, followed by a rapidly declining secondary phase, and return to baseline level within 80 s (Della Penna *et al.*, 2003; Goold *et al.*, 2001). This increase in $[Ca^{2+}]_i$ persists in a calcium-free medium suggesting that the increase in $[Ca^{2+}]_i$ in response to TIP39 comes largely from intracellular stores. The potency of TIP39 is lower for increases of $[Ca^{2+}]_i$ than for increases of cAMP suggesting that the PTH2R is more weakly coupled to increasing $[Ca^{2+}]_i$ than to increasing cAMP accumulation (Goold *et al.*, 2001). In addition, the signal transduction pathway of the PTH2R depends on the agonist.

Although the human PTH2R can be activated both by TIP39 and PTH (as described below), the resulting cAMP and intracellular Ca signaling is different (Bisello *et al.*, 2004). PTH stimulation of cAMP formation is brief and rapidly resensitizes, the response to TIP39 is sustained and remains partly desensitized for a prolonged period. Furthermore, TIP39 induces beta-arrestin and protein kinase C-beta mobilization, PTH2R desensitization, and receptor internalization whereas PTH does not (Bisello *et al.*, 2004). The effect of PTH on the PTH2R is also different from that on the PTH1R, where PTH promotes phospholipase C/PKC activation, arrestin trafficking, receptor endocytosis, and desensitization of both Gs- and Gq-signaling (Castro *et al.*, 2002; Dicker *et al.*, 1999; Ferrari *et al.*, 1999; Ferrari and Bisello, 2001). These findings suggest that PTH and TIP39 may play distinct physiological activities through the human PTH2R. However, the rat PTH2R is poorly activated by PTH (Hoare *et al.*, 1999) and it is not clear whether PTH reaches brain PTH2Rs, so PTH may not be a physiological agonist of the PTH2R.

6.3 TIP39 interaction with PTH receptors

TIP39 was purified on the basis of its stimulation of cAMP accumulation in HEK293 cells transfected with the PTH2R (Usdin *et al.*, 1999b). Subsequent experiments demonstrated that TIP39 is a high potency specific ligand for the PTH2R. Human (identical to bovine) TIP39 (hTIP39) had essentially no activity at the human or rat PTH1R expressed in COS-7 or HEK293 cells (Hansen *et al.*, 2002; Usdin *et al.*, 1999b). Furthermore, while hTIP39 is a potent agonist of the zebrafish PTH2R, it produced no activation of the zebrafish PTH1R or PTH3R. Nevertheless, hTIP39 was found to bind the human PTH1R receptor with moderate affinity in PTH1R expressing LLCPK1 (Jonsson *et al.*, 2001), and COS-7 cells (Hoare *et al.*, 2000b) as well as in isolated membranes prepared from transfected COS-7 cells (Hoare *et al.*, 2000a). In addition, hTIP39 has a moderate affinity for the zebrafish PTH3R approximately equal to that at the human PTH1R (Hoare *et al.*, 2000b). Based on the binding of TIP39 to the PTH1R, the possibility that it may be an endogenous antagonist has been raised (Jonsson *et al.*, 2001), but this does not seem likely because of the relatively low affinity binding of TIP39 to the PTH1R and their different tissue localizations.

6.4 Agonist pharmacology of the PTH2R

Comparison of the effect of human (identical to bovine) TIP39 (hTIP39), rat (identical to mouse) TIP39 (rTIP39), hPTH(1-34), rPTH(1-34), and hPTHrP on cAMP formation, as well as on intracellular calcium levels in cell lines stably expressing the hPTH2R or the rPTH2R (Goold *et al.*, 2001) is shown in Table 1. hTIP39 at 1 μ M concentration evoked a robust response in both assays and both receptors. hPTH(1-34) has a greater EC50 and smaller maximal effect than hTIP39 at the hPTH2R, making it a weak partial agonist, and it has almost no effect at the rPTH2R. rPTH(1-34) was also less potent than hTIP39 but was a near-full agonist at the hPTH2R, while also having almost no effect at the rPTH2R. Human and rat TIP39 have similar potency and efficacy at the rat and human PTH2Rs (Goold *et al.*, 2001). In addition, PTHrP (1-34) did not activate either the human or rat PTH2Rs and PTH displayed a lower efficacy for increasing intracellular calcium levels *via* the hPTH2R than for the elevation of cAMP accumulation (Della Penna *et al.*, 2003; Goold *et al.*, 2001). Pharmacological data from several studies and several groups of investigators using various combinations of intracellular calcium and cAMP assays, and receptors and cell lines, is consistent (Della Penna *et al.*, 2003; Goold *et al.*, 2001; Hoare *et al.*, 1999; Hoare *et al.*, 2000a; John *et al.*, 2002; Usdin *et al.*, 1999b). Similar to mammalian PTH2Rs, the zebrafish PTH2R (zPTH2R) is potently activated by zTIP39 as well as by hTIP39 while rPTH and hPTH are only partial agonists of the zPTH2R (Hoare *et al.*, 2000b; Papasani *et al.*, 2004).

Ligand binding to human and rat PTH2Rs is consistent with the functional assays. hTIP39 and rTIP39 have slightly higher affinity for displacement of 125I-hTIP39 binding to COS-7 cells

expressing the hPTH2R than to cells expressing the rat receptor, and rTIP39 has slightly higher affinity than hTIP39 for both PTH2Rs (Goold *et al.*, 2001). The hPTH2R has somewhat greater selectivity for rPTH(1-34) over hPTH(1-34) and both ligands are significantly weaker at the rPTH2R (Table 1).

6.5 Molecular determinants of ligand-receptor interaction for the PTH2R

Structure–activity studies have generated a model for the interaction of PTH(1–34) and PTHrP (1–34) with the PTH1 receptor: contact of N-terminal residues of either peptide with the juxtamembrane ‘body’ of the PTH1R is responsible for receptor activation, and interactions of C-terminal peptide sequences with the extracellular N-terminus of the receptor contribute substantially to high-affinity binding (Mannstadt *et al.*, 1999). Discrimination between PTH and PTHrP by the hPTH2R has allowed function to be assigned to individual peptide residues. Replacement of residue 23 of PTH with residue 23 of PTHrP prevents binding of PTH to the hPTH2R, whereas transfer of residue 5 from PTHrP to PTH limits the efficacy of PTH (Gardella *et al.*, 1996). Corresponding modifications of PTHrP facilitate its binding to, or activation of, the PTH2R (Gardella *et al.*, 1996). These data support the N-terminal-activation, C-terminal binding model and have extended it to the PTH2R. Consistent with this model, a chimeric receptor composed of the N-terminal extracellular domain of the PTH1R and the remainder (juxtamembrane domain) of the PTH2R was fully activated by TIP39 (Hoare *et al.*, 2000a). This receptor chimera bound TIP39 with an equivalent affinity to the wild-type PTH2R. The reciprocal chimeric receptor was not activated by TIP39 and bound the ligand with an affinity equivalent to that of the PTH1R (Hoare *et al.*, 2000a). Thus, the juxtamembrane receptor domain specifies the signaling and binding selectivity of TIP39 for the PTH2R over the PTH1R. Removing six N-terminal residues of TIP39 eliminated activation of the PTH2R and reduced the binding affinity 70-fold. In contrast, this truncation increased affinity for the PTH1R 10-fold, reversing the PTH2/PTH1R binding selectivity and resulting in a high affinity interaction of TIP(7–39) with the PTH1R (Goold *et al.*, 2001; Hoare *et al.*, 2000a; Jonsson *et al.*, 2001). These findings can be explained by a strong interaction between the N-terminal region of TIP39 and the juxtamembrane domain of the PTH2R, with the corresponding domain of the PTH1R acting as a selectivity barrier against high affinity binding of TIP39. These data are also consistent with the finding that residues that are the same in TIP39, PTH(1–34) and PTHrP(1–36) are within the carboxyl part of these peptides, the region that is responsible for high-affinity receptor binding, as opposed to the N-termini, which are crucial for receptor activation (Hoare and Usdin, 2001; Juppner *et al.*, 1991).

6.6 Antagonists of the PTH2R

Deletion of residues from the amino terminus of PTH or PTHrP reduces their ability to activate the PTH1 receptor while having relatively small effects on their binding affinity. PTH(7-34), PTHrP(7-36), and several analogs of these peptides are PTH1R antagonists (Gardella *et al.*, 1996). Similar modifications of TIP39 were produced in attempts to generate a PTH2R antagonist. Removing amino terminal residues from TIP39 did reduce its ability to activate the PTH2R, but it also reduced its affinity for the PTH2R such that a peptide with no detectable agonist activity, TIP(7-39), had too low an affinity to be useful as a PTH2R antagonist (Hoare *et al.*, 2000a). Surprisingly, removing amino terminus residues from TIP39 increased its affinity for the PTH1R, and TIP(7-39) (Hoare and Usdin, 2000) and TIP(9-39) (Jonsson *et al.*, 2001) are potent PTH1R antagonists.

In a further attempt to develop a PTH2R antagonist, the effect of changing some of the N-terminal residues to ones with different charge or bulk was examined (Kuo and Usdin, 2007). Several potent and selective PTH2R antagonists were developed. One, His4, Tyr5, Trp6, His7-TIP39 (HYWH-TIP39), is particularly promising based on its potency and selectivity. In cultured cells HYWH-TIP39 completely blocks activation of the rPTH2R by TIP39 and has

over 30-fold selectivity for the rPTH2R over the rPTH1R (Kuo and Usdin, 2007). The major potential use for HYWH-TIP39 is to facilitate the investigation of TIP39's physiological role by using it within in vivo experiments. Although its refractoriness to peptidase inactivation and its penetration through the blood brain barrier are not known, it is likely to be a useful reagent for investigation of the TIP39-PTH2R system when delivered through intracerebral cannulae.

A novel technology for monitoring the changes in cAMP levels in live cells has recently been applied for screening small-molecule antagonists of the PTH2R (Visegrady *et al.*, 2007). Cyclic nucleotide-gated channels as biosensors were coexpressed with the PTH2R. The technique allowed the measurement of cAMP-dependent calcium influx or membrane depolarization with conventional fluorescent methods in both kinetic and in endpoint modes for high-throughput and subsequent compound screening (Visegrady *et al.*, 2007). This high throughput screen has identified a potential lead compound with potential utility as PTH2R ligands (Visegrady *et al.*, 2007).

7 PTH2R expressing cell groups

The PTH1R is most abundant in kidney and bone where PTH exerts its calcium regulatory functions and it is also present several other organs (Hurwitz, 1996; Rizzoli *et al.*, 1992). In contrast, the PTH2R is not highly expressed in kidney or bone, and has a limited expression pattern in peripheral organs (Usdin *et al.*, 1996; Usdin *et al.*, 1999a). The PTH2R is most abundantly expressed in the central nervous system (Usdin *et al.*, 1995) where it has a more widespread and profoundly different expression pattern (Faber *et al.*, 2007; Wang *et al.*, 2000) than the PTH1R whose mRNA has been demonstrated in the anterodorsal nucleus of the thalamus, the basolateral amygdala, entorhinal cortex, parasubiculum, the mesencephalic nucleus of the trigeminal nerve, the motor nucleus of the trigeminal and the facial nerve, the lateral reticular, pontine and reticulotegmental nuclei, vestibular nuclei, the ventral cochlear nucleus, the hypoglossal nucleus and the area postrema, Purkinje cells, and the Gasserian ganglion (Weaver *et al.*, 1995).

7.1 Peripheral distribution and actions of TIP39 and the PTH2R

As evaluated on Northern blots PTH2R mRNA is most abundant in the brain and is detected in the testis and pancreas (Usdin *et al.*, 1995). *In situ* hybridization histochemistry (Usdin *et al.*, 1996) and immunohistochemistry (Usdin *et al.*, 1999a) demonstrate expression of the PTH2R in arterial endothelium, which is most clear in large vessels in bronchi and the parenchyma in the lung, in cardiac endothelium, in a small number of cells associated with the vascular pole of renal glomeruli, in the pancreas, in spermatids in the head of the epididymis, in atretic follicles of the ovary, in chondrocytes in thyroid cartilage, in a small number of cells in bone, and in some endocrine cells including thyroid parafollicular cells, and some gastrointestinal peptide synthesizing cells. Since TIP39 has not been reported to be present in the blood, these receptors might be activated by locally produced TIP39. Existing data on the function of PTH2R's in peripheral organs is only briefly summarized, as most of them are likely independent of the neural functions of the TIP39-PTH2R neuromodulator system.

In testis, TIP39 and the PTH2R are expressed in a stage-specific manner within seminiferous tubules (Usdin *et al.*, 2008) and TIP39 expression is greatest in mature testes. Mice with deletion of the gene encoding TIP39 are sterile (Usdin *et al.*, 2008). Testes contained Leydig and Sertoli cells and spermatogonia but no spermatids, because spermatogonia did not complete prophase of meiosis I. In addition, in mice expressing Cre recombinase under control of a spermatid-specific promoter to selectively induce TIP39 expression in the testes of TIP39^{-/-} mice, spermatid production and fertility were rescued. These data demonstrate that

TIP39 is essential for germ cell development and suggest that it may act as an autocrine or paracrine agent within the gonads (Usdin *et al.*, 2008).

TIP39 and the PTH2R are also implicated in the regulation of renal hemodynamics. TIP39 and PTH2R mRNA are expressed in rat intrarenal arteries as well as in renovascular smooth muscle cells cultured from these arteries (Eichinger *et al.*, 2002). TIP39 has complex vasoactivity. At 10 nM it constricts and at 1, 100, and 1000 nM it dilates isolated perfused rat kidneys that have been desensitized to the vasodilatory action of PTHrP (1-36).

In addition to the PTH2R expression in the heart described above, TIP39 mRNA is also constitutively expressed in coronary endothelium, isolated cardiomyocytes, in the wall of the ventricles and atria, and in the aorta (Ross *et al.*, 2005). Inotropic actions of TIP39 are observed in Langendorff perfused rat hearts (Ross *et al.*, 2005). TIP39 influenced the contractility of the rat heart *via* the PTH2R in two different and contrary ways: a positive inotropism mediated by activation of the nitric oxide signaling pathway and a negative inotropism *via* a yet unknown pathway (Ross *et al.*, 2005). Furthermore, there appears to be an interaction between PTH1R and PTH2R (Ross *et al.*, 2007). Ischemia-dependent endogenously released PTHrP desensitizes the PTH1R, which enables activation of the PTH2R by TIP39. PTH2R activation dilates vessels in a nitrogen oxide-dependent manner (Ross *et al.*, 2007). In contrast to these data, intravenous TIP39 had no effect on blood pressure or heart rate in anesthetized mice and no differences were observed between wild-type and TIP39 knockout mice in a number of measures of cardiovascular function at baseline (Fegley *et al.*, 2008).

A number of peripheral endocrine cells express PTH2Rs (Usdin *et al.*, 1999a). In the thyroid gland PTH2Rs are not observed in the follicular cells but are clearly present on parafollicular C cells. PTH2R labeling partially overlaps somatostatin labeling of C cells demonstrating that only a subpopulation of parafollicular cells express the PTH2R. Expression by these calcitonin synthesizing cells potentially places the PTH2R in a calcium regulating pathway.

All pancreatic islet somatostatin synthesizing D cells are intensely labeled by a PTH2R selective antibody, and none of the other populations of islet cells have detectable immunostaining (Usdin *et al.*, 1999a). This highly selective expression of PTH2Rs on pancreatic islet somatostatin cells suggests that it may modulate somatostatin synthesis or release and thus indirectly affect glucagon or insulin release.

7.2 Distribution of neurons expressing PTH2R in the nervous system

PTH2R expression in the nervous system has been investigated by a great variety of techniques. *In situ* hybridization histochemistry was used to directly visualize the presence of RNA in the rat nervous system (Wang *et al.*, 2000), in the mouse brain (Faber *et al.*, 2007), and in the macaque brainstem (Bagó *et al.*, 2009) while RT-PCR indicated its expression in several regions of the human brain (Bagó *et al.*, 2009). A mouse line has been developed in which the first coding exon of the PTH2R gene is replaced with the bacterial lacZ coding sequence (Faber *et al.*, 2007). In these knockin mice beta-galactosidase (coded for by the lacZ gene) activity can be detected histochemically in cells in which the PTH2R promoter is active (Faber *et al.*, 2007). The two different methods indicated the same distribution for PTH2R-expressing cells in virtually all brain areas, and this distribution was the same in male and female mice (Faber *et al.*, 2007). In contrast, immunolabeling of PTH2R cell bodies in mouse and human was not reliable. Few and only lightly labeled cell bodies were found, if any at all, in most brain regions (Bagó *et al.*, 2009; Faber *et al.*, 2007). In contrast, the immunolabeling of PTH2R neurons was relatively satisfactory in the rat as demonstrated by the same distribution of the PTH2R *in situ* hybridization signal and the PTH2R-ir cell bodies in most brain regions (Wang *et al.*, 2000). A potential explanation for this finding may be that the transport of PTH2R protein out of cell bodies and into neuronal fibers is faster in mice and human than in rats. The distribution

of PTH2R-expressing neurons in the mouse brain obtained by *in situ* hybridization as well by beta-galactosidase histochemistry in the PTH2R-lacZ knockin line, and a comparison with the distribution of PTH2R neurons in mice to that in other species is shown in Fig. 10.

In the cerebral cortex, PTH2R-expressing neurons are widely distributed with a density that varies somewhat between regions. In the limbic system, there are a few PTH2R-expressing neurons in the anterior olfactory nucleus and in the olfactory tubercle. In the septum, only the lateral nucleus contains PTH2R-expressing neurons. This region contains a very high density of PTH2R-expressing neurons, especially in its ventral part. More rostrally, the intermediate part of the lateral septal nucleus is also rich in PTH2R neurons. Most subdivisions of the bed nucleus of the stria terminalis contain a moderate density of PTH2R-expressing neurons, in contrast to the posteromedial part of the medial subdivision where the density is very high. Many PTH2R-expressing neurons are present in the amygdala. The density of PTH2R-expressing neurons is high in the medial nucleus, especially in its posterodorsal subdivision while it is moderate in the anterior amygdaloid area, the central and cortical amygdaloid nuclei, and the amygdala-hippocampal transitional zone. A low density of scattered PTH2R-expressing neurons is present throughout the hippocampal CA areas and a slightly higher in the dentate gyrus and the subiculum. The claustrum and the dorsal endopiriform nucleus contain a fairly large number of PTH2R-expressing neurons, while there is a moderate density of randomly distributed PTH2R-expressing neurons throughout the accumbens nucleus, the caudate-putamen, the ventral striatum, and in the substantia innominata. The thalamus is relatively poor in PTH2R-expressing neurons except for a few regions. The medial subdivision of the medial geniculate body contains a very high density of PTH2R-expressing neurons, whereas it is moderate in the ventral subdivision. There is a high to moderate density of PTH2R-expressing neurons in some midline and intralaminar thalamic nuclei. In addition, a moderate density of PTH2R-expressing neurons is present in the epithalamic lateral habenular nucleus and the subthalamic zona incerta. Finally, beta-galactosidase histochemical reaction product is present in the dorsal part of the lateral geniculate nucleus of PTH2R knockin mice, but *in situ* hybridization for the PTH2R does not indicate PTH2R-expressing neurons in this brain area. PTH2R-expressing neurons are abundant in many regions of the hypothalamus. In the preoptic region, a high number of PTH2R-expressing neurons is present in the medial preoptic nucleus, whereas there is a low density of PTH2R-expressing neurons in other parts of the medial preoptic area. In the anterior hypothalamic region, a moderate density of PTH2R-expressing neurons is present in the paraventricular and periventricular nuclei, while the anterior hypothalamic nucleus contains a low density of PTH2R-expressing neurons. In the middle portion of the hypothalamus, a high density of PTH2R-expressing neurons is present in the arcuate nucleus, whereas a moderate density is observed in the dorsomedial and perifornical hypothalamic nuclei, and some parts of the lateral hypothalamic area including the so-called far-lateral hypothalamus (Forel's field) immediately next to the internal capsule. In the posterior hypothalamus, a high density of PTH2R-expressing neurons is seen in the medial subdivision of the supramammillary nucleus while its lateral subdivision, and the ventral premammillary, and the tuberomammillary nuclei contain a moderate density of PTH2R-expressing neurons. In contrast, the medial and lateral nuclei of the mammillary body do not contain PTH2R-expressing neurons. There are relatively few PTH2R-expressing neurons in the lower brainstem and cerebellum. The midbrain contains a moderate density of PTH2R-expressing neurons in the lateral interpeduncular, paranigral, and median raphe nuclei, and there are a few scattered PTH2R-expressing neurons in the superior and inferior colliculi. In the pons, there is a moderate to high density of PTH2R-expressing neurons in the sphenoid nucleus of the tegmental area, and in the nucleus of the trapezoid body. In addition, beta-galactosidase (but not *in situ* hybridization signal) was present in the ventral cochlear nuclei. In the medulla oblongata, the nucleus of the solitary tract contains a moderate density, and the spinal trigeminal nucleus a low density of PTH2R-expressing neurons. A few scattered PTH2R-expressing

neurons are present in the cerebellar cortex, with a somewhat higher density in the superficial portion of the molecular layer.

The distribution of PTH2R-expressing cell bodies in mice is similar to that of rats (Wang *et al.*, 2000) in most brain regions, including the cerebral cortex, the caudate-putamen, the claustrum and the endopiriform nucleus, the lateral septum, the hippocampus, the amygdala, and many thalamic, hypothalamic, and brainstem regions, and the cerebellum. In some brain regions, however, marked differences were found in the density of PTH2R-expressing neurons between mice and rats. Most particularly, a strong *in situ* hybridization signal but no more than a few immunoreactive cell bodies are found in the medial septal, the ventromedial hypothalamic, the parabrachial, and the spinal trigeminal nuclei in rats (Wang *et al.*, 2000). None of these regions contain a significant number of PTH2R-expressing cells in mice. In addition, both *in situ* hybridization histochemistry and immunocytochemistry reveal a high density of PTH2R-expressing neurons in the periventricular hypothalamic nucleus in rat, while this area contains only a moderate number of PTH2R-expressing neurons in mice. In contrast, there are brain regions that are abundant in PTH2R-expressing neurons in mice but not in rats, including the posteromedial part of the medial division of the bed nucleus of the stria terminalis, the medial geniculate body, and the nucleus of the trapezoid body.

The overall distribution of PTH2R mRNA in human and macaque is similar to that in rodents (Bagó *et al.*, 2009). A detailed mapping of PTH2R mRNA expression by *in situ* hybridization histochemistry has only been performed in the macaque and the regional and subregional distribution is very similar to that of rodents (Bagó *et al.*, 2009). Nevertheless, there are two regions, namely the ventral part of the lateral hypothalamus and the parabigeminal nucleus where PTH2R-expressing cells formed compact cell groups in the macaque while only scattered labeled cells appear in rodents, suggesting a relatively small species difference (Bagó *et al.*, 2009).

PTH2R expression in the spinal cord and sensory ganglia has only been described in the rat. *In situ* hybridization histochemistry shows numerous PTH2R-positive cells in the marginal layers of the dorsal horn and the lateral cervical nucleus, as well as in dorsal root ganglia, and a few labeled cells in the deep layers of the dorsal horn of the spinal cord (Dobolyi *et al.*, 2002; Wang *et al.*, 2000).

7.3 The distribution of PTH2R fibers and their topographical correlation with TIP39 fibers

PTH2R-immunoreactive fibers are widely distributed in the rat nervous system (Wang *et al.*, 2000), the rat hypothalamus (Dobolyi *et al.*, 2006a), the mouse brain (Faber *et al.*, 2007), and in the human brainstem (Bagó *et al.*, 2009). Strikingly, the distribution of PTH2R-containing fibers is very similar to the distribution of TIP39-containing fibers (see Fig. 10 and Fig. 11B,C, as an example). In addition, no difference was found between males and females in their distribution of PTH2R-containing or TIP39-containing fibers (Faber *et al.*, 2007). The distribution of PTH2R-containing fibers in rat and mouse and, to the extent mapped, in human is almost identical. The few differences may be due to different immunolabeling techniques since no differences were found in the hypothalamus when the same amplification technique was applied (Dobolyi *et al.*, 2006a; Faber *et al.*, 2007). Therefore, we present the distributions of TIP39 and PTH2R fibers only in mice and compare them with each other as well as to the distribution of PTH2R-expressing neurons in Table 2.

7.4 The TIP39-PTH2R neuromodulator system

The localization of cell bodies that express TIP39 and those that express the PTH2R are profoundly different. TIP39 expression is confined to the subparafascicular area-posterior intralaminar thalamic complex and the medial paralemniscal nucleus, while the PTH2R is

expressed in many brain regions. In general, TIP39-ir neurons have axons that project long distances from the TIP39-expressing perikarya, while PTH2R-ir fibers are often localized in the vicinity of PTH2R-expressing neurons (Fig. 11A,B). Although the origin of PTH2R-ir fibers have not yet been investigated in any brain region, it seems logical that they are of local origin. In contrast to the profoundly different localization of TIP39- and PTH2R-expressing cell bodies, the distributions of TIP39-ir and PTH2R-ir fibers are markedly similar. TIP39-ir and PTH2R-ir fibers are present in the same nuclei and areas throughout the central nervous system except for a few areas described below. Furthermore, TIP39-ir and PTH2R-ir fibers show topographical similarities not only in certain brain regions, but very often also in subdivisions (Fig. 11B,C). In fact, the two distributions are indistinguishable in most brain nuclei and areas. This finding suggests the probable action of TIP39 on the PTH2R in these brain regions. Together with the strong pharmacological evidence that TIP39 is a potent and high-affinity selective ligand for the PTH2R, the anatomical data also support that TIP39 is the endogenous ligand for the PTH2R in the brain. Based on the signal transduction mechanisms used by TIP39 and the PTH2R they may form a neuromodulator system in many brain regions.

In a few areas, including the median eminence, the interpeduncular, and the spinal trigeminal nuclei, as well as the superficial laminae of the spinal cord dorsal horn PTH2R-ir but not TIP39-ir fibers are abundant. This could be explained by the appearance of TIP39 in these areas under specific physiological conditions, although a nonfunctional expression of the PTH2R or the existence of another ligand for the PTH2R in these areas cannot be excluded. It is possible that circulating TIP39 acts on PTH2R's in the median eminence, but a likely source of that TIP39 has not been identified. Mismatches between the distributions of neuropeptides and their putative receptors have also been explained by the ability of peptides that have high affinity for their receptors to act at a very low concentration, potentially after diffusing long distances, as well as by the presence of peptide or receptor in some axonal or dendritic branches in which they do not have functional relevance (Herkenham, 1987; Leng and Ludwig, 2006).

7.5 The glutamatergic nature of PTH2R fibers and a proposed model for the neuromodulatory action of TIP39

Glutamate is the major excitatory neurotransmitter in the central nervous system. Its quantal release depends on its transport into synaptic vesicles. Therefore, glutamatergic terminals contain vesicular glutamate transporters (VGLUT), which are generally used as a marker of glutamatergic terminals (Freneau *et al.*, 2001). VGLUT-2 is the major VGLUT form in the hypothalamus and the septum (Lin *et al.*, 2003). Most, or all, PTH2R-containing terminals contain VGLUT-2 in the rat hypothalamus (Dobolyi *et al.*, 2006a) and macaque's septum and hypothalamus (Bagó *et al.*, 2009). The co-localization of PTH2R and VGLUT-2 in fiber terminals suggests that PTH2R-containing neurons in the septum and hypothalamus are excitatory glutamatergic neurons. Glutamic acid decarboxylase, the synthetic enzyme for GABA (Erlander and Tobin, 1991) is also abundant in the hypothalamus (Vincent *et al.*, 1982). Consistent with PTH2R-containing neurons being glutamatergic and not GABAergic, however, no glutamic acid decarboxylase was co-localized with the PTH2R in the hypothalamus (Dobolyi *et al.*, 2006a). TIP39 terminals do not contain either VGLUT-2 or glutamic acid decarboxylase immunoreactivity.

The excellent correlation between PTH2R- and TIP39-ir fibers and fiber terminals together with the finding that PTH2R immunoreactivity is not consistently detectable in cell bodies suggests that TIP39 and PTH2R may be involved in axo-axonal interactions. Both TIP39-ir and PTH2-ir fiber terminals are abundant in the medial preoptic, paraventricular and arcuate nuclei (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007) where the perikarya of the vast majority of the hypothalamic neurosecretory neurons are located (Palkovits, 1992). Neurosecretory cells

are likely to be regulated by VGLUT-containing excitatory synapses, as demonstrated for the gonadotropin-releasing hormone-containing cells in the medial preoptic area (Kiss *et al.*, 2003). Assuming that excitatory synapses regulating neurosecretory cells contain PTH2Rs, TIP39 terminals are ideally situated to modulate these synapses. This hypothesis may explain how TIP39 regulates the secretion of hypophysiotropic hormones, i.e. an axo-axonal model (Fig. 12) could represent the general mechanism of actions for the TIP39-PTH2R neuromodulator system.

8 Central regulatory mechanisms affected by TIP39

8.1 Nociceptive information processing

There is intense PTH2R immunoreactivity in superficial layers of the spinal cord dorsal horn where most nociceptive afferents terminate (Wang *et al.*, 2000). This has led to evaluation of the role of TIP39 in the modulation of spinal nociceptive information transfer. Intrathecal injection of TIP39 stimulates a dose-dependent nocifensive response, caudally directed scratching, biting, and licking (Dobolyi *et al.*, 2002). Intrathecal TIP39 administration also decreased the tail-flick and paw-pressure withdrawal latencies, but did not have a significant effect on withdrawal in response to paw heating. Furthermore, microinjection of TIP39 into the plantar surface of a mouse paw elicited a dose-dependent withdrawal response. These responses to peripheral and central TIP39 administration show that it can activate nociceptive circuits. However, these observations do not address the effects of endogenous TIP39. Because no TIP39 antagonists were available at the time of these experiments, TIP39 was sequestered with an antibody followed by testing nociceptive responses (Dobolyi *et al.*, 2002). Intrathecal injection of the TIP39 antibody increases the response latency in the thermal tail-flick assay. In the paw-pressure test, intrathecal administration of the TIP39 antibody increases the latency to paw withdrawal, corresponding to decreased pressure sensitivity. In contrast, intrathecal delivery of the TIP39 antibody does not significantly affect paw withdrawal latency in response to radiant heat in the Hargreaves' assay (Hargreaves *et al.*, 1988) and it does not affect the response to intraplantar injection of the chemoirritant capsaicin (Dobolyi *et al.*, 2002). Inhibition by the TIP39 antibody of responses in the thermal tail-flick and paw-pressure assays suggests that TIP39 may have a facilitatory role in the underlying circuits. The tail-flick response is thought to be primarily a spinal reflex, whereas paw withdrawal from heat requires supraspinal mediation. The different effects of TIP39 and TIP39 sequestration on these responses may reflect differences in PTH2R expression on neurons in the underlying circuits. However, the intrathecally applied reagents may also reach relevant sites with different efficiency.

To identify the neuronal circuitry responsible for the nociceptive actions of TIP39 in the spinal cord, PTH2R-ir fibers were double labeled with established markers of dorsal horn populations, including CGRP and substance P, as well as isolectin B4, markers of small-diameter primary afferent fibers terminating in the marginal layers, (Molliver *et al.*, 1995; Snider and McMahon, 1998), and protein kinase C gamma, which is synthesized in the spinal cord dorsal horn and labels fibers in lamina III (Malmberg *et al.*, 1997; Polgar *et al.*, 1999). PTH2R labeling showed extensive laminar overlap with isolectin B4, it partially overlapped but was mostly ventral to the area of greatest substance P and CGRP labeling (marginal layers), and dorsal to the layer of labeling by an antibody to protein kinase C gamma (lamina III) placing the PTH2R within lamina II (Dobolyi *et al.*, 2002), which contains most of the central terminals of nociceptive primary afferents. Despite the laminar overlap, PTH2R did not co-localize with isolectin B4 or substance P. There was, however, limited co-localization with CGRP fibers (Dobolyi *et al.*, 2002). The PTH2R is synthesized by a population of dorsal root ganglion (DRG) cells, which seemed to be of the smaller size class (Dobolyi *et al.*, 2002). In addition, TIP39 increased cAMP in F-11 cells (Usdin *et al.*, 1999b), which are a DRG-neuroblastoma hybrid cell line that possesses some of the properties of peptidergic nociceptors (Kusano and Gainer, 1993).

Other agents that increase cAMP in DRG neurons potentiate nociception (Taiwo *et al.*, 1989). PTH2R expressing cells are also present within the dorsal horn of the spinal cord (Wang *et al.*, 2000), while relatively little PTH2R synthesis occurs in supraspinal areas that project to the superficial dorsal horn (Wang *et al.*, 2000). Thus, PTH2R fibers in the superficial laminae of the spinal cord dorsal horn may originate in the DRG and/or the spinal cord dorsal horn.

TIP39 mRNA is not detected in the spinal cord by RT-PCR. RT-PCR does suggest that there may be a low level of TIP39 synthesis in DRG, but TIP39 has not been detected in DRG by immunocytochemistry or *in situ* hybridization histochemistry (Dobolyi *et al.*, 2002). TIP39 acting on PTH2Rs in the superficial laminae may have a supraspinal origin. Neurons within areas expressing TIP39 including the PVG (Björklund and Skagerberg, 1979; Hökfelt *et al.*, 1979; Skagerberg and Lindvall, 1985) and the PIL (Basbaum and Fields, 1979; Carlton *et al.*, 1985; Holstege and Kuypers, 1982) project to the dorsal horn of the spinal cord. Most TIP39 fibers are in the lateral funiculus adjacent to the gray matter, an area that contains both descending and primary afferent fibers. In the spinal cord, fine scattered TIP39-ir fibers are found in the lateral funiculus, the lateral intermediate gray matter, central gray matter, and lamina II and III. As described above, studies using microstimulation and correlation with electrical activity implicate the PVG (Dong *et al.*, 1978; Peschanski and Mantyh, 1983; Rhodes and Liebeskind, 1978; Sugiyama *et al.*, 1992) and the MPL (Basbaum *et al.*, 1977; Girardot *et al.*, 1987; Hardy *et al.*, 1983; Haws *et al.*, 1989; Zhao and Duggan, 1988) in nociception. Thus, it can be hypothesized that descending TIP39 fibers may contribute to modulation of spinal nociceptive information.

TIP39 appears to have another effect on nociception in the brain (LaBuda and Usdin, 2004). In adult male rats i.c.v. TIP39 does not change hot-plate paw withdrawal latency or formalin test behavioral responses. TIP39 partially reverses tactile withdrawal hypersensitivity following carrageenan administration. In the place/escape avoidance paradigm, which evaluates affective components of the response to noxious stimuli by presenting a choice between a naturally preferred environment paired with stimulation of a carrageenan sensitized paw and a less preferred environment paired with stimulation of a less sensitive paw, TIP39 decreases the apparent aversiveness of sensitive paw stimulation (LaBuda and Usdin, 2004). Because acute sensory thresholds are unaffected by i.c.v. TIP39, and effects in some nociceptive tests are opposite in direction from effects of intrathecal TIP39, this suggests that TIP39 has distinct brain and spinal roles. It may modulate an affective component of nociception within the brain. The TIP39-PTH2R neuromodulator system is also potentially involved in the processing of nociceptive and viscerosensory input to the limbic system, which is suggested by its presence in brain areas such as the nucleus of the solitary tract, the parabrachial nuclei, the midline thalamic nuclei, the paraventricular hypothalamic nucleus, and the insular and infralimbic cortex that are components of the autonomic-limbic pain-related pathway (Benarroch, 2006).

8.2 Affective processes modulated by TIP39

Several of the forebrain limbic regions that contain TIP39 and the PTH2R are implicated in anxiety and depression-related neuronal circuits (Drevets, 2000; Kent and Rauch, 2003; Manji *et al.*, 2001). Therefore TIP39's effects were evaluated in tests of anxiety- and depression-like behaviors following its i.c.v. administration in rat (LaBuda *et al.*, 2004) and by evaluating behavior changes in mice with targeted null mutation of the TIP39 gene (Tip39^{-/-}) or TIP39-KO mice (Fegley *et al.*, 2008). TIP39-KO mice cannot be distinguished from wild-type (WT) littermates on casual observation or in a functional observational battery for general health, reflexes, and basic sensory and motor functions. There were also no significant differences between the genotypes in home cage locomotor activity over 72 hours of monitoring, in motor coordination assessed on an accelerating rotarod or in exploration in a holeboard test. Thus, TIP39-KO mice do not show any gross phenotypic abnormalities that are likely to confound

performance on tests for anxiety-like behaviors. TIP39-KO mice did not differ significantly from WT littermates in the open field test (Fegley *et al.*, 2008). Furthermore, i.c.v. TIP39 administration in rat did not significantly modify any of the behaviors evaluated in the open field including total number of partitions entered over a 5 min test, number of rearing incidents, number of fecal boli, or bouts of grooming (LaBuda *et al.*, 2004). At the time of the rat experiments, there were no high-affinity or specific PTH2R antagonists so the effects of several doses of injected TIP39 were compared with those of the truncated analog TIP(7-39) to help evaluate the specificity of the effect of TIP39 injection in rat behavioral experiments. As described above, TIP(7-39) is a very weak PTH2R antagonist (Hoare *et al.*, 2000a). Animals receiving TIP(7-39) did not differ from control animals on any of the tests performed (LaBuda *et al.*, 2004).

8.2.1 Elevated plus maze test—The elevated plus maze is one of the most commonly used tests of anxiety-like behavior in rodents. Animals are allowed to explore a maze that consists of two open and two enclosed arms. The relative amount of time spent in the two conditions is considered an index of the anxiety state of the animal. Anxiolytic agents increase the relative amount of time that an animal spends exploring the open arms.

Following i.c.v. administration of TIP39, rats entered the open arms more frequently and spent more time within the open arms as compared to controls (animals receiving TIP(7-39) or saline) while no differences were observed between groups in the number of closed arm entries or total arm entries suggesting an anxiolytic-like effect of TIP39 (LaBuda *et al.*, 2004). In contrast, under “standard” testing conditions, TIP39-KO mice do not have robust differences from WT controls in several tests of spontaneous anxiety-like behaviors including the novel open field, dark-light emergence and elevated plus maze (Fegley *et al.*, 2008). However, an increase in anxiety-like behavior becomes apparent in TIP39-KO mice that are tested under conditions of increased stress. Either following brief restraint, or under bright illumination, TIP39-KO mice made fewer open arm entries and spent less time in the open arms than WT littermates.

8.2.2 Shock-probe defensive burying test—In the shock-probe burying test animals receive a shock if they contact an electrified rod placed in their cage. This is thought to produce an approach/avoid conflict somewhat like the elevated plus maze, but with a stronger, tangible, aversive stimulus (i.e., shock) than an exploration-based assay (Sluyter *et al.*, 1996; Treit and Fundytus, 1988). Animals typically respond by avoiding the area closest to the rod and by actively pushing bedding on top of it.

TIP39-KO mice spent more time engaged in defensive burying than WT controls in the shock-probe burying test (Fegley *et al.*, 2008). KO mice also spent significantly less time in the zone around the electrified probe. This response is similar to their response in the elevated plus maze in that it demonstrates more anxiety-like behavior by the TIP39-KO mice, and it complements it by showing an active response.

8.2.3 Forced swim test—The forced swim test is commonly used with rodents to evaluate depression-like behavior. Animals are placed in a cylinder of water and the amount of time they spend passively floating vs. actively attempting to escape is measured. Established antidepressant agents decrease the relative amount of time spent passively floating.

I.c.v. administration of TIP39 reduced the duration of immobility, and increased the amount of climbing behavior in the forced swim test (LaBuda *et al.*, 2004). TIP(7-39), administered as a control, had no effect on swimming behavior.

8.2.4 Pavlovian fear conditioning and extinction—Administration of paired tone and shock in a specific context, followed at later times by exposure to either the original context,

or to the tone alone in a novel context, is often used to establish and evaluate conditioning in rodents, with freezing used as an index of fear to measure the strength of the conditioning (Blanchard and Blanchard, 1969; Bouton and Bolles, 1979; LeDoux *et al.*, 1984).

Pavlovian fear conditioning and its extinction were evaluated in TIP39-KO mice (Fegley *et al.*, 2008). During conditioning, TIP39-KO mice froze significantly more than WT controls during the second but not first or third tone presentation (Fegley *et al.*, 2008). During a tone test performed 24 hours later in a novel context, TIP39-KO mice froze significantly more than WT controls when the tone was presented, but not during the pre-tone period. TIP39-KO mice also froze significantly more than WT controls when placed in the original training context without tone stimulation (Fegley *et al.*, 2008).

Fear extinction was also assessed in TIP39-KO and WT mice. In this experiment TIP39-KO mice again had greater freezing scores than WT controls during the second (but not first or third) tone-shock pairing during conditioning (Fegley *et al.*, 2008). During extinction training TIP39-KO mice initially froze more than WT controls. With repeated tone presentations WT and TIP39-KO mice had parallel reductions in freezing, such that there was no difference between the groups by the end of the session. Freezing did not differ between the genotypes during extinction recall, and WT and KO mice re-extinguished at the same rate over the remainder of the recall session (Fegley *et al.*, 2008).

8.2.5 The role of TIP39 in affective brain regulatory processes—Antidepressant- and anxiolytic-like effects of TIP39 as well as the increase in anxiety-like behavior in the shock-probe burying test and in the elevated plus maze observed in TIP39-KO mice are consistent with an anxiolytic role of endogenous TIP39. Behavioral effects of TIP39 absence were only apparent in the elevated plus maze when the stress levels of the tests were increased slightly, by prior brief restraint or using bright illumination. This suggests that the role of endogenous TIP39 may involve modulation of the response to stress. In these studies, the stressors that evoked increased anxiety-like behaviors in TIP39-KO mice were in themselves insufficient to produce a significant anxiety-like response in WT controls, suggesting that endogenous TIP39 may play a role in the presence of relatively mild perturbations. The manifestation of a clear phenotype in the shock-probe burying test even without prior stress might be due to the inherently higher level of stress and anxiety-provocation in this shock-based assay (Holmes *et al.*, 2005).

In addition to increased innate anxiety-like behaviors under stress, TIP39-KO mice showed enhanced acquisition and recall of conditioned fear responses. KOs showed more freezing than WT controls after only one tone-shock pairing during conditioning and, subsequently, more freezing during both tone- and context-recall tests. However, based on a similar rate of decline in freezing responses to repeated tone presentation and a similar level of freezing during subsequent tone presentation, there did not appear to be an effect of TIP39 deletion on fear extinction learning or extinction recall, respectively.

The behavioral observations suggest that endogenous TIP39 may modulate fear and stress-induced anxiety and/or reduce the behavioral manifestation of fear and stress following environmental provocation. This is consistent with growing evidence that neuropeptides are preferentially recruited as modulators of fear and anxiety under conditions of stress (Holmes *et al.*, 2003; Hökfelt *et al.*, 2003). Data on TIP39 support the hypothesis that TIP39 modulates anxiety-related behaviors.

8.2.6 Sites of TIP39 effect on affective behaviors—There is a high density and quite restricted distribution of TIP39-positive fibers and PTH2Rs (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007) in regions known to mediate fear and anxiety, including parts of the amygdala, bed

nucleus of the stria terminalis, lateral septum, and the medial prefrontal cortex (Drevets, 2000; Kent and Rauch, 2003; Manji *et al.*, 2001). In an attempt to further specify the sites of action of TIP39 within the forebrain, the pattern of *c-fos* expression was examined following i.c.v. delivery of TIP39. Administration of TIP39 into the lateral ventricle elicited robust increases in the number of *c-fos*-expressing cells in five brain regions, i.e. in the infralimbic cortex, the lateral hypothalamus, the preoptic area, the lateral septum, and in the paraventricular thalamic nucleus (LaBuda *et al.*, 2004). As shown previously, relatively high PTH2R levels were found in all of these areas. Thus, induction of *c-fos* expression in these brain areas may indicate that TIP39 directly activates these cells *via* PTH2Rs. The lack of *c-fos* activation in other PTH2R expressing cells could be a consequence of different intracellular events following PTH2R activation, or insufficient diffusion of TIP39 to those sites from the lateral ventricle. However, the *c-fos* activation pattern might be elicited by some more complex events. Anxiogenic drugs with four different mechanisms of action produce a similar *c-fos* activation pattern, suggesting their influence on a common fear circuitry (Singewald *et al.*, 2003). Neurons in all above listed five TIP39-activated regions also exhibit *c-fos* expression in response to different stressors. In particular, exposure to the elevated plus maze induces *c-fos* activation in the infralimbic cortex, in the lateral septum and in the lateral hypothalamus (Campeau *et al.*, 1997; Duncan *et al.*, 1996; Pezzone *et al.*, 1992). Data about *c-fos* activation together with the neuroanatomical distribution of the PTH2R and TIP39 suggest that limbic regions described above may represent the sites where TIP39 may act to modulate affective or anxiety state.

8.3 Neuroendocrine actions of TIP39 at hypothalamic level

The TIP39-PTH2R neuromodulator system is in optimal position to influence the hypothalamo-pituitary axis through its presence in several hypothalamic areas. Initial functional data suggest that TIP39 may influence the release of adrenocorticotropin (ACTH), luteinizing hormone (LH), growth hormone (GH), and arginine-vasopressin (AVP) from the pituitary.

8.3.1 Hypothalamo-pituitary-adrenal axis—Plasma ACTH increased following i.c.v. administration of TIP39 (Ward *et al.*, 2001). In addition, there was a robust release of corticotropin releasing hormone (CRH) from medial basal hypothalamic explants following incubation with TIP39 (Ward *et al.*, 2001). Therefore, the increased ACTH following i.c.v. delivery of TIP39 could be mediated *via* stimulation of hypothalamic CRH release. Considering the presence of PTH2Rs in the PVN and the external zone of the median eminence (Dobolyi *et al.*, 2006a; Faber *et al.*, 2007; Wang *et al.*, 2000), the projection of TIP39 neurons to the hypothalamic paraventricular nucleus (Dobolyi *et al.*, 2003a; Palkovits *et al.*, 2004) and the *in vitro* and *in vivo* effects of TIP39, these data suggest an activating role for TIP39 in the hypothalamo-pituitary-adrenal axis.

8.3.2 Hypothalamo-pituitary-gonadal axis—Plasma LH also increased with i.c.v. administration of TIP39 (Ward *et al.*, 2001). In addition, luteinizing hormone releasing hormone (LHRH) increased in the media of hypothalamic explants following incubation with TIP39 (Ward *et al.*, 2001), suggesting that the increase in plasma LH may be mediated by LHRH. However, TIP39 administered intraperitoneally (ip) also resulted in a significant release of LH 10 min after the injection, compared with saline (Ward *et al.*, 2001). In light of this rapid response of LH to peripheral TIP39, a direct pituitary action is possible. Although PTH2R mRNA was detected in anterior pituitary cells (Usdin *et al.*, 1996), the PTH2R was not demonstrated by immunohistochemistry in the anterior pituitary, probably on account of its low concentration (Usdin *et al.*, 1999a). In addition, TIP39 does not alter the release of any anterior pituitary hormone from monodispersed anterior pituitary cells (Ward *et al.*, 2001). However, the PTH2R is present in the external zone of the median eminence (Dobolyi *et al.*,

2006a; Faber *et al.*, 2007; Wang *et al.*, 2000), where it could mediate the release of hypothalamic LHRH. While further investigation is required to identify the site of action of TIP39 on the hypothalamo-pituitary-gonadal axis, the combined results suggest that TIP39 may contribute to the regulation of the LH secretion, potentially acting both directly and indirectly on the hypothalamo-pituitary-gonadal axis.

8.3.3 Regulation of the secretion of growth hormone—In rat, both *in situ* hybridization and immunohistochemistry show PTH2R expression by cell bodies in the hypothalamic periventricular nucleus. This area contains somatostatin neurons that project to the median eminence and contribute to the regulation of growth hormone secretion. Double labeling shows a high degree of co-localization of somatostatin and the PTH2R in periventricular neurons (Usdin *et al.*, 1999b), and PTH2R fibers in the median eminence in rats (Dobolyi *et al.*, 2006a), and also in human (Bagó *et al.*, 2009). These anatomical findings suggest that the PTH2R might be involved in the control of GH secretion. In one experiment, TIP39 injection into the lateral ventricle of male rats blocked the appearance of GH almost completely in plasma for the next three hours (Usdin *et al.*, 2003). This is consistent with the suggestion that TIP39 stimulates release of somatostatin, which in turn inhibits GH secretion. However, another study found no alteration in plasma GH following i.c.v. administration of TIP39 and no effect of TIP39 on somatostatin release from hypothalamic explants (Ward *et al.*, 2001).

8.3.4 Arginine-vasopressin release—The effect of centrally administered TIP39 on AVP release was investigated in conscious rats (Sugimura *et al.*, 2003). Plasma AVP levels were significantly lower 5 min following i.c.v. administration of TIP39 as compared with controls (Sugimura *et al.*, 2003). TIP39 did not significantly change plasma Na⁺ or plasma total protein levels measured for estimation of the change in plasma volume. TIP39 also significantly suppressed the dehydration-induced plasma AVP increase, with its maximum effect occurring 5 min after injection in rats, water deprived for 48 h. Furthermore, the plasma AVP increase in response to either hyperosmolality following i.p. injection of hypertonic saline or hypovolemia following i.p. injection of polyethylene glycol was also significantly attenuated 5 min after i.c.v. TIP39 injection (Sugimura *et al.*, 2003). These inhibitory effects cannot be attributed to a decrease in the level of osmotic or hypovolemic stimulation because plasma Na⁺ and plasma total protein were not affected by the i.c.v. injection of TIP39. In addition, i.c.v. injection of TIP39 produced a fall in mean arterial blood pressure, which is well known to stimulate AVP secretion suggesting that the inhibitory effect of TIP39 was not based on the change in blood pressure (Sugimura *et al.*, 2003). Taken together, these results suggest that TIP39 inhibits AVP release by central action, without hemodynamic or osmotic alterations. The effect was rapid and did not last long suggesting that TIP39 may play a role in the dynamic regulation of AVP release.

TIP39 and PTH2R are particularly abundant in the hypothalamic periventricular and arcuate nuclei and are scarce in the supraoptic nucleus and the magnocellular part of the hypothalamic paraventricular nucleus where AVP is synthesized. Thus, it seems likely that TIP39 exerts its AVP release-inhibitory effect by acting through other hypothalamic nuclei. The arcuate nucleus is a strong candidate. It contains many opioid neurons (Palkovits, 1988), and a great deal of evidence suggests that opioid systems are involved in the regulation of AVP release (Haaf *et al.*, 1987; Van de Heijning *et al.*, 1991a; Van de Heijning *et al.*, 1991b). Most studies demonstrate that opioids play an inhibitory role in the regulation of AVP release, and that such a release in response to hyperosmolality or hypovolemia is attenuated by opioid agonists (Haaf *et al.*, 1987; Oiso *et al.*, 1988). Because of the massive neural connections between arcuate and the hypothalamic paraventricular nuclei (Baker and Herkenham, 1995; Bell *et al.*, 2000), the possibility that TIP39 suppression of AVP release is mediated by opioids was investigated by co-administration of naloxone. Naloxone significantly reversed the inhibitory effect of TIP39

on dehydration-induced AVP release whereas naloxone alone did not significantly alter the plasma AVP level (Sugimura *et al.*, 2003). Together, these findings suggest that TIP39 plays an inhibitory role in the osmoregulation and baroregulation of AVP release, and that intrinsic opioid systems are involved in the action of TIP39.

8.3.5 The role of TIP39 in the secretion of hypophysiotropic hormones—

Experiments investigating the effect of TIP39 on the release of hypothalamic hormones suggest the possibility that TIP39 plays a role in regulating the hypothalamo-pituitary axis, although most of the results must be considered preliminary. First, relatively large doses of peptide were administered in these experiments. These high concentrations might be required because of the need for tissue penetration and the occurrence of proteolysis, as the peptide diffuses through tissue. In addition, dose-response studies and experiments to test the mechanism of action of TIP39's effects on the release of hypothalamic and pituitary hormones were often not performed. It is also clear that manipulation of the effect of endogenous TIP39 is essential to understand its physiological relevance, which requires studies, in which the effects of TIP39 are antagonized. In addition, the effects of a single injection of TIP39 were usually examined, which may not be a good mimic of endogenous TIP39's effect. Little is known about the regulation of TIP39 release or synthesis, and further studies are required to clarify this point, as well.

Based on the pharmacology and anatomy of the TIP39-PTH2R system, it is likely that TIP39 effects are mediated by the PTH2R. However, somatostatin is the only neuroendocrine factor shown yet to co-localize with the PTH2R (Bagó *et al.*, 2009; Dobolyi *et al.*, 2006a), suggesting that TIP39 might directly regulate the secretion of somatostatin but not other hypophysiotropic hormones. As discussed above, there is a high degree of co-localization between VGLUT2 and the PTH2R in the hypothalamus so TIP39 may indirectly regulate other neurosecretory cells *via* effects at VGLUT2-containing (glutamatergic) excitatory synapses, which contain PTH2R in their terminals.

8.4 Modulatory action of TIP39 on the auditory system

The TIP39-PTH2R neuromodulator system may also be involved in the processing of auditory information because it is present in several auditory brain centers including the auditory cortex, the medial geniculate body and surrounding 'auditory thalamic areas', the external cortex of the inferior colliculus, the superior olive, and the nucleus of the trapezoid body (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007). Both neurons and fibers containing TIP39 or PTH2R are located in non-tonotopically organized regions (Clopton *et al.*, 1974). Therefore, we propose that the TIP39-PTH2R neuromodulator system may modulate auditory processes that do not require tonotopic information such as loud noise-induced stress response or acoustic fear-conditioning. Such potential functions of the TIP39-PTH2R neuromodulator system are supported by the loud noise-induced activation in posterior intralaminar and medial paralemnisal TIP39 neurons (Palkovits *et al.*, 2004, 2009a) and their significant neuronal input from the auditory cortex (Varga *et al.*, 2008). Auditory stimulation (30 min 105 dB white noise) elicits *c-fos* expression in the majority of TIP39-ir neurons in the medial paralemnisal nucleus (Fig. 13C, D), in about half of TIP39-ir neurons in the PIL (Fig. 13E), and about 20% of TIP39-ir neurons in the subparafascicular nucleus (Fig. 13F). The participation of TIP39 neurons in loud noise-induced stress response is also supported by tracer studies demonstrating that parvicellular subparafascicular and medial paralemnisal neurons project to the area of the hypothalamic paraventricular nucleus that contains the CRH synthesizing neurons, and may therefore represent an interconnection between the auditory system and the stress center of the hypothalamus (Palkovits *et al.*, 2004). Similarly, the projection of TIP39 neurons to the amygdala (Dobolyi *et al.*, 2003b) supports their involvement in acoustic fear-conditioning (LeDoux, 2003).

8.5 Involvement of TIP39 neurons in central reproductive regulations

The most established function of TIP39 in peripheral organs is the role in the development of the gonads described above (Usdin *et al.*, 2008). TIP39 and the PTH2R are present in several brain areas involved in some aspect of reproduction, and the developmental expression pattern of TIP39 and the apparent sexual dimorphism in adult TIP39 levels are consistent with a role of the TIP39-PTH2R neuromodulator system in some aspects of reproductive function.

8.5.1 A potential role of TIP39 in male sexual behaviors—The role of the TIP39-PTH2R neuromodulator system in male sexual behaviors is supported by the presence of PTH2R as well as TIP39 fibers in several brain areas that are activated in males following mating (Coolen *et al.*, 1997; Sachs and Meisel, 1988; Veening and Coolen, 1998) including the medial preoptic nucleus, the posteromedial part of the medial subdivision of the bed nucleus of the stria terminalis, and the posterodorsal subdivision of the medial amygdaloid nuclei. In addition, the medial subdivision of the PIL, which has been implicated in sexual functions by stimulation (Shimura and Shimokochi, 1991), lesion (Maillard and Edwards, 1991), and activation studies (Coolen *et al.*, 1997; Holstege *et al.*, 2003) is one of the 3 brain sites that contain TIP39-expressing neurons. Indeed, TIP39 neurons in the PIL have been shown to exhibit *c-fos* activation following ejaculation, and much less following intromission without ejaculation (Wang *et al.*, 2006a). This suggests that these TIP39 neurons are part of the afferent circuits that process genital-somatosensory information related to ejaculation contributing to mating and mating-induced changes in reproductive behaviors. Based on the visceral, somatosensory, proprioceptive, and noxious information that can reach the PIL (Coolen *et al.*, 2003b), TIP39 neurons in the area are in position to integrate and transmit information related to copulation.

8.5.2 Topography of the TIP39-PTH2R system and changes in TIP39 expression in relation to central reproductive functions—An interconnected network of brain centers regulates behavioral, emotional, and endocrinological influences on reproductive physiology. It has been suggested that limbic, preoptic and hypothalamic areas, including the lateral septum, the bed nucleus of the stria terminalis, the medial preoptic nucleus and area, the anteroventral periventricular, paraventricular and ventromedial hypothalamic nuclei, the arcuate nucleus, the medial and central amygdaloid nuclei, the amygdalo-hippocampal transitional zone, the premamillary nuclei, the ventral subiculum, and the periaqueductal gray, are part of the circuitry of reproductive regulation (Gorski, 1985; Hasen and Gammie, 2005; Holstege *et al.*, 2003; Li *et al.*, 1999; Lin *et al.*, 1998; Lonstein and Stern, 1997; Numan and Insel, 2003; Numan and Sheehan, 1997; Pfaus and Heeb, 1997; Sachs and Meisel, 1988; Simerly, 2002; Veening and Coolen, 1998). The TIP39-PTH2R neuromodulator system is abundant in all of these brain regions (Dobolyi *et al.*, 2003b; Faber *et al.*, 2007) suggesting its involvement in reproductive regulation.

TIP39 levels increase and then decrease during development as described in Chapter 3.7. In the amygdalo-hippocampal transitional zone and in most of the neurons in the PIL, TIP39 disappears during early postnatal development (Brenner *et al.*, 2008; Dobolyi *et al.*, 2006b). As TIP39 can affect the proliferation of some cell types in vitro (Misiano *et al.*, 2003), it is possible that TIP39 might be involved in aspects of neural development including brain sexual development during its transient expression. In addition, TIP39 gradually disappears from neurons in the PVG and the MPL during the period of pubertal development (Dobolyi *et al.*, 2006b), which is consistent with a potential role of TIP39 related to pubertal development.

9 Conclusion and future directions in the research on the TIP39-PTH2R system

TIP39 and the PTH2R form a unique neuropeptide-receptor system. Its signal transduction pathways suggest that it is a neuromodulator and its anatomical distribution suggests that it plays a role in limbic, endocrine, viscerosensory, and auditory functions. Mice with targeted mutations of the TIP39-PTH2R system and specific antagonists have recently become available and these tools should facilitate identification of the specific roles of TIP39 and the PTH2R. The TIP39-PTH2R system may be worth considering as a therapeutic target as it differs neuroanatomically and functionally from other receptor systems that have been proposed as potential drug targets in anxiety, depression, and chronic pain management.

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Abbreviations

ACTH	adrenocorticotropin
AVP	arginine-vasopressin
CGRP	calcitonin gene-related peptide
CRH	corticotropin releasing hormone

DRG	dorsal root ganglion
ED	embryonic day
GH	growth hormone
i.c.v.	intracerebroventricular
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
MPL	medial paralemniscal nucleus
PIL	posterior intralaminar complex of the thalamus
PND	postnatal day
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
PTH1R	PTH 1 receptor
PTH2R	PTH 2 receptor
PTH3R	PTH 3 receptor
PVG	periventricular gray of the thalamus
SPF	subparafascicular area
SPFp	parvicellular subparafascicular thalamic nucleus
TH	tyrosine hydroxylase
TIP39	tuberoinfundibular peptide of 39 residues
TIP39-KO	mice with targeted null mutation of the TIP39 gene
VGLUT	vesicular glutamate transporter
WT	wild type

Fig. 1.
Schematic structure of the mRNA encoding TIP39.

Fig. 2.

TIP39 neurons in the subparafascicular area of the rat posterior thalamus. **A:** TIP39-ir neurons (green) are shown in the PVG in coronal sections labeled with a fluorescent Nissl dye (red) at antero-posterior (AP) coordinates -4.2, -4.4, and -4.6 mm from the bregma level. **B:** The position of the PVG is shown in a drawing of a sagittal section of the rat brain at 0.4 mm lateral from the midline (Paxinos and Watson, 1998). **C:** A photomicrograph of a sagittal section corresponding to panel B shows the location of TIP39-ir neurons in the PVG. **D:** TIP39-ir neurons and fibers are shown in a horizontal section 6.8 mm ventral to the surface of the brain. The large density of TIP39-ir neurons in the PVG is in contrast to the scattered TIP39-ir neurons in the PIL while TIP39-ir fibers connect the 2 regions of TIP39 expression. *Abbreviations:* ac, anterior commissure; AH, anterior hypothalamic nucleus; cc, corpus callosum; CM, central median thalamic nucleus; Dk, nucleus of Darkschewitsch; DpMc, deep mesencephalic nucleus; f, fornix; fr, fasciculus retroflexus; IC, inferior colliculus; IPF, interpeduncular fossa; LS,

lateral septal nucleus; ml, medial lemniscus; MPA, medial preoptic area; mt, mamillothalamic tract; ox, optic chiasm; PAG, periventricular gray; pc, posterior commissure; PF, parafascicular thalamic nucleus; PH, posterior hypothalamic area; PIN, posterior intralaminar thalamic nucleus; Pn, pontine nuclei; PVG, periventricular gray of the thalamus; py, pyramidal tract; Re, reuniens thalamic nucleus; RI, rostral interstitial nucleus of the medial longitudinal fasciculus; SC, superior colliculus; SPFm, magnocellular subparafascicular thalamic nucleus; SPFp, parvicellular subparafascicular thalamic nucleus; Sub, submedial thalamic nucleus; VPM, ventral posteromedial thalamic nucleus; VPPC, ventral posterior parvicellular thalamic nucleus; ZI, zona incerta; 3V, third ventricle; 4V, fourth ventricle. Scale bar = 500 μ m for A and D, and 300 μ m for C.

Fig. 3.

The posterior intralaminar complex of the thalamus (PIL). Schematic drawings of a horizontal section 6.8 mm ventral to the surface of the brain (Paxinos and Watson, 1998) show the topographical position and the parts of the PIL. The framed area on the left is magnified on the right. Red dots represent the location of TIP39 neurons in the medial part of the PIL.

Abbreviations: ac, anterior commissure; ic, internal capsule; Cb, cerebellum; CP, caudate-putamen; DG, dentate gyrus; DpMe, deep mesencephalic nucleus; EW, Edinger-Westphal nucleus; fr, fasciculus retroflexus; ml, medial lemniscus; mlf, medial longitudinal fasciculus; opt, optic tract; PIL, posterior intralaminar complex of the thalamus; PIN, posterior intralaminar thalamic nucleus; PP, peripeduncular nucleus; SPFp, parvicellular subparafascicular thalamic nucleus; SubB, subbrachial nucleus; Tg, tegmental nuclei; VPM, ventral posteromedial thalamic nucleus; VPPC, ventral posterior parvocellular thalamic nucleus; ZI, zona incerta; 3V, third ventricle.

Fig. 4.

The medial paralemniscal nucleus (MPL) in the rat. **A:** The position of TIP39 neurons (black arrows) is shown in a coronal section stained with a combination of Luxol dye to visualize myelinated fibers in blue and cresyl-violet to label cell bodies. **B:** The MPL is delineated by the distribution of TIP39 neurons (green) in a section labeled with fluorescent Nissle dye (red) at 8.3 mm caudal to the bregma level. **C:** A high magnification photomicrograph demonstrates the dorsolaterally oriented columnar organization of the MPL. **D:** A schematic drawing of a horizontal section 7.8 mm ventral to the surface of the brain (Paxinos and Watson, 1998) shows the position of the MPL by a black arrow. **E:** A photomicrograph of a horizontal section corresponding to the position of the framed area in D demonstrated the position of the MPL by TIP39-ir (green). **F:** The same area in a double immunolabeled section shows the relation of TIP39 neurons (green) in the MPL and TH (red) neurons belonging to the A7 noradrenergic cell group. Panels B, C, and E are modifications of our previously published figures (Varga *et al.*, 2008). *Abbreviations:* A7, A7 noradrenaline cell group; ILL, intermediate nucleus of the lateral lemniscus; KF, Kölliker-Fuse nucleus; mcp, middle cerebellar peduncle; MPL, medial paralemniscal nucleus; M5, motor nucleus of the trigeminal nerve; PnO, oral part of the pontine reticular nucleus; PPTg, pedunculopontine tegmental nucleus; RR, retrorubral nucleus; rs, rubrospinal tract; VLL, ventral nucleus of the lateral lemniscus; 4V, fourth ventricle. Scale bar = 500 μ m for B, E, and F, and 100 μ m for C.

Fig. 5.

The summary of brain areas providing major neuronal inputs to TIP39-expressing brain regions. The PVG and its neuronal inputs are indicated by blue, the PIL and its inputs by green, and the MPL and its inputs by red arrows. *Abbreviations:* ac, anterior commissure; AH, anterior hypothalamic nucleus; Amy, amygdala; Aud Cx, auditory cortex; BNST, bed nucleus of the stria terminalis; cc, corpus callosum; cic, commissure of the inferior colliculus; CnF, cuneiform nucleus; DHA, dorsal hypothalamic area; Ect, ectothalamic cortex; f, fornix; fr, fasciculus retroflexus; IC, inferior colliculus; IL, infralimbic cortex; Ins, insular cortex; LH, lateral hypothalamic area; LS, lateral septal nucleus; ml, medial lemniscus; MPL, medial paralemniscal nucleus, mt, mamillothalamic tract; NTS, nucleus of the solitary tract; ox, optic chiasm; PAG, periventricular gray; PB, parabrachial nuclei; pc, posterior commissure; PIL, posterior intralaminar complex of the thalamus; POA, preoptic area; PrL, prelimbic cortex; PVG, periventricular gray of the thalamus; PVN, hypothalamic paraventricular nucleus; py, pyramidal tract; S, subiculum; SC, superior colliculus; SI, substantia innominata; SPA, superior periolivary nuclei, Sp5, spinal trigeminal nucleus; VMH, hypothalamic ventromedial nucleus; 3V, third ventricle; 4V, fourth ventricle.

Fig. 6.

Demonstration of projections from the auditory cortex to the MPL in the rat. **A:** Injection site of the retrograde tracer cholera toxin beta subunit (red) in the MPL identified by TIP39-ir neurons (green). **B:** Retrogradely labeled neurons are visible in layer V of the primary auditory cortex and the ventral secondary auditory cortex. A lower density of labeled cells are present in layers Vi and V of the perirhinal cortex and layer VI of the ventral secondary auditory cortex. **C:** BDA was injected into the primary and secondary auditory cortices along the same pipette tract. **E:** In the same brain, anterogradely labeled fibers (green) are distributed among TIP39-ir neurons (red) in the MPL. The panels are modifications of our previously published figures (Varga *et al.*, 2008). *Abbreviations:* Au1, primary auditory cortex; AuV, ventral part of the secondary auditory cortex; CP, caudate-putamen; ic, internal capsule; ILL, intermediate nucleus of the lateral lemniscus; LV, lateral ventricle; MPL, medial paralemniscal nucleus; PnO, oral part of the pontine reticular nucleus; PRh, perirhinal cortex; rs, rubrospinal tract; scp, superior cerebellar peduncle. Scale bar = 500 μ m for A, B, and C, and 300 μ m for D.

Fig. 7.

The localization of TIP39-ir fibers in the diencephalon, and the amygdala. The photomicrograph demonstrates the topographically organized distribution of TIP39-ir fibers in a coronal section of the rat brain at the level of the hypothalamic ventromedial nucleus. *Abbreviations:* Arc, arcuate nucleus; BLA, basolateral amygdaloid nucleus; BMA, basomedial amygdaloid nucleus; CeA, central amygdaloid nucleus; CP, caudate-putamen; DMH, hypothalamic dorsomedial nucleus; f, fornix; FS, fundus striati; LH, lateral hypothalamic area; LV, lateral ventricle; MeA, medial amygdaloid nucleus; ot, optic tract; PVT, paraventricular thalamic nucleus; sod, supraoptic decussations; VB, ventrobasal thalamic nuclei; VMH, hypothalamic ventromedial nucleus; ZI, zona incerta; 3V, third ventricle. Scale bar = 1 mm.

Fig. 8.

The disappearance of TIP39 fibers following lesioning TIP39 neurons in the rat. **A:** Unilateral lesions of the PVG resulted in the ipsilateral disappearance of TIP39 fibers in the lateral septal nucleus. **B:** The density of TIP39-ir fibers is markedly reduced in the hypothalamic paraventricular nucleus ipsilateral to a lesion in the PIL. **C:** The dense network of TIP39 fibers in the external cortex of the inferior colliculus disappears ipsilateral to a lesion of the MPL. Panels B, C, and E are modifications of our previously published figures (Dobolyi *et al.*, 2003a). *Abbreviations:* ac, anterior commissure; f, fornix; IC, inferior colliculus; LS, lateral septal nucleus; LV, lateral ventricle; PVN, hypothalamic paraventricular nucleus; 3V, third ventricle; 4V, fourth ventricle. Scale bar = 400 μ m for A, 200 μ m for B, and 500 μ m for C.

Fig. 9.

The summary of the major projections of TIP39 neurons. The PVG and the projections of its TIP39 neurons are indicated by blue, the PIL and the projections of its TIP39 neurons by green, and the MPL and the projections of its TIP39 neurons by red arrows. *Abbreviations:* ac, anterior commissure; AH, anterior hypothalamic nucleus; Amy, amygdala; BNST, bed nucleus of the stria terminalis; cc, corpus callosum; cic, commissure of the inferior colliculus; CnF, cuneiform nucleus; DMH, hypothalamic dorsomedial nucleus; Ect, ectorhinal cortex; f, fornix; fr, fasciculus retroflexus; IC, inferior colliculus; IL, infralimbic cortex; Ins, insular cortex; LH, lateral hypothalamic area; LS, lateral septal nucleus; ml, medial lemniscus; MPL, medial paralemniscal nucleus; mt, mamillothalamic tract; ox, optic chiasm; PAG, periventricular gray; PB, parabrachial nuclei; pc, posterior commissure; PIL, posterior intralaminar complex of the thalamus; POA, preoptic area; PrL, prelimbic cortex; PVG, periventricular gray of the thalamus; PVN, hypothalamic paraventricular nucleus; PVT, thalamic paraventricular nucleus; py, pyramidal tract; S, subiculum; SC, superior colliculus; SI, substantia innominata; SPA, superior periolivary nuclei; SpC, spinal cord; VMH, hypothalamic ventromedial nucleus; 3V, third ventricle; 4V, fourth ventricle.

Fig. 10.

Schematic diagrams demonstrate the distribution of TIP39 (left side) and the PTH2R (right side) in the brain of mice. The diagrams are modifications from a mouse stereotaxic atlas (Franklin and Paxinos, 1997). Dots represent fibers and fiber terminals, while squares represent cell bodies. The figure is a slight modification of our previously published figure (Faber *et al.*, 2007).

Fig. 11.

Comparison of PTH2R-expressing neurons, PTH2R-, and TIP39-immunoreactivities in the preoptic region of mice. **A:** PTH2R-expressing neurons are present in the posteromedial part of the medial subdivision of the bed nucleus of the stria terminalis (arrows) and the medial preoptic nucleus, as demonstrated by X-gal histochemistry in PTH2R knockin mice. **B:** The distribution of PTH2R-ir fibers is similar to the distribution of PTH2R-expressing neurons. **C:** The distribution of TIP39-ir fibers looks exactly the same as the distribution of PTH2R-ir fibers in the preoptic region of mice. The panels are modifications of our previously published figures (Faber *et al.*, 2007). *Abbreviations:* BST, bed nucleus of the stria terminalis; f, fornix; MPN, medial preoptic nucleus; 3V, third ventricle. Scale bar = 500 μm.

Fig. 12.

A model on the modulatory action of TIP39. TIP39 neurons located in the PVG, PIL, and MPL project to remote brain areas where their axon terminals approach PTH2R-containing terminals. As demonstrated in the hypothalamus and the septum, PTH2R are present in glutamatergic terminals. It was hypothesized that TIP39 is released from the terminals of axons originating in the PVG, PIL, and MPL, and presynaptically modulates the efficiency of excitatory synaptic transmission by acting on PTH2Rs in the glutamatergic axon terminals.

Fig. 13.

Activation of TIP39 neurons in response to acute loud noise in the rat. **A:** The dorsal as well as the ventral cochlear nuclei demonstrate *c-fos* activation following exposure to 30-min of 105 dB white noise. **B:** The density of neurons expressing Fos is high in the auditory cortex but not in the somatosensory cortex. The caudal portion of the caudate-putamen also contains a great number of Fos-expressing neurons. **C:** Fos-ir neurons are abundant in the inferior colliculus, the nuclei of the lateral lemniscus, as well as the medial paralemniscal nucleus. TIP-ir neurons (brown) can be seen at low power. **D, E, F:** High magnification photomicrographs of double immunolabeled sections demonstrate that a portion of TIP39-ir neurons (brown cell bodies) express Fos (black nuclei) in response to acute loud noise. The percentage of double

labeled TIP39 neurons is very high in the MPL (D), somewhat lower in the PIL (E), and relatively modest in the PVG (F). *Abbreviations:* AudCx, auditory cortex; CP, caudate-putamen; DCN, dorsal cochlear nucleus, ec, external capsule; fi, fimbria hippocampi, ic, internal capsule, IC, inferior colliculus; ll, lateral lemniscus; m, midline; MPL, medial paralemniscal nucleus; ot, optic tract; PIL, posterior intralaminar complex of the thalamus, PVG, periventricular gray of the thalamus, py, pyramidal tract; rf, rhinal fissure; rs, rubrospinal tract; SomCx, somatosensory cortex; VCN, ventral cochlear nucleus; VPM, ventral posteromedial thalamic nucleus, 4V, fourth ventricle. Scale bar = 1 mm for A, B, C, 200 μ m for F, and 100 μ m for D, and E.

Table 1
Agonist pharmacology of human and rat PTH2 receptors expressed in COS-7 cells.

agonist peptides	hPTH2R				rPTH2R			
	[Ca ²⁺] _i		cAMP		[Ca ²⁺] _i		cAMP	
	EC ₅₀ , nM	E _{max} , % of hTIP39	EC ₅₀ , nM	E _{max} , % of hTIP39	EC ₅₀ , nM	E _{max} , % of hTIP39	EC ₅₀ , nM	E _{max} , % of hTIP39
hTIP39	3.8	100	0.6	100	15	100	1.5	100
mTIP39	1.8	108±7	0.5	97±6	2.3	113±6	0.5	89±6
hPTH(1-34)	22	44±6	1.7	99±13		4±8	93	32±2
rPTH(1-34)	16	81±7	0.2	118±13		7±8	37	38±5
PTHrP(1-34)		5±9				12±2		
					850			5100

Agonist concentration dependence relationships were measured as the increase in intracellular calcium summed over a 60-s time course, cAMP accumulation measured after 40-min incubations with the ligands. Receptor binding was determined by displacement of 125I-TIP39 binding. The table is a slight modification of our previously published table (Goold *et al.*, 2001).

Table 2

Location of PTH2R perikarya and fibers, as well as TIP39 fibers in mice brain.

Area	PTH2Rcell bodies	PTH2R-ir fibers	TIP39-ir fibers
Forebrain Cerebral cortex			
Medial prefrontal cx.	+	++	++
Somatomotor cortex	+	0	0
Somatosensory cortex	+	0	0
Insular cortex	++	+	+
Ectorhinal cortex	+	+	++
Temporal cortex	+	0	0
Hippocampus	+	0	0
Subiculum	+	+	++
Septum			
Medial septal nucleus	0	0	0
Lateral septal nucleus	+++	+++	+++
Bed nu. stria terminalis	+	++	+++
Amygdala			
“Fundus striati”	0	+	++
Central nucleus	++	++	++
Basal nuclei	+	0	0
Lateral nucleus	+	0	0
Medial nucleus	++	++	++
Cortical nucleus	+	+	0
Amygdalo-hipp. trans.	0	0	+
Basal ganglia			
Caudate nucleus	++	0	0
Globus pallidus	+	0	0
Clastrum	++	+	+
Nucleus accumbens	+	++	++
Substantia innominata	+	+	++
Diencephalon Thalamus			
Anterior thalamic nu.	0	0	0
Midline thalamic nu.	++	++	++
Lateral thalamic nuclei	0	+	+
Ventral thalamic nuclei	0	0	0
Reticular nucleus	0	0	0
Mediodorsal th. nu.	0	0	0
Habenular nuclei	+	+	+
Posterior th. nuclei	0	+	+
Peripeduncular area	+	+	+
Suprageniculate th. nu.	+	+	++
Medial geniculate body			
dorsal nucleus	0	0	0

Area	PTH2Rcell bodies	PTH2R-ir fibers	TIP39-ir fibers
ventral nucleus	++	+	0
medial nucleus	+++	++	+
Lateral geniculate body	+	+	0
Hypothalamus			
Medial preoptic area	+++	+++	+++
Lateral preoptic area	+	+	+
Supraoptic nucleus	+	++	+
Supraoptic decuss.		++	+++
Suprachiasmatic nu.	0	0	0
Anterior hypoth. nu.	+	+	+
Paraventricular nucleus	++	+++	+++
Periventricular nucleus	++	++	++
Arcuate nucleus	++	+++	+++
Median eminence		+++	+
Ventromedial nucleus	+	+	+
Dorsomedial nucleus	+	++	+++
Lateral hypoth. area	+	++	++
Perifornical nucleus	+	+	+
Posterior hypoth. nu.	+	++	++
Tuberomamillary nu.	+	++	++
Premamillary nuclei	+	++	++
Supramamillary nucleus	++	++	++
Mamillary body			
Medial mamillary nu.	0	0	0
Lateral mamillary nu.	0	++	++
Subthalamic nucleus	+	+	+
Brainstem Midbrain			
Zona incerta	++	+++	+++
Periaqueductal gray	++	+++	+++
Ventral tegmental area	++	+++	++
Oculomotor nuclei	0	0	0
Substantia nigra	0	0	0
Red nucleus	0	0	0
Subbrachial nucleus	+	++	++
Praetectal area	+	++	++
Superior colliculus	++	++	++
Inferior colliculus	+	++	++
Dorsal raphe nucleus	++	+++	++
Pons			
Lateral lemniscal nuclei	0	0	0
Medial paralemniscal nu.	0	+	++
Pontine tegmentum	++	++	++

Area	PTH2Rcell bodies	PTH2R-ir fibers	TIP39-ir fibers
Parabrachial nuclei	+		
medial		+	+
lateral		+++	+++
Pontine nuclei	0	0	0
Superior olive	0	+	++
Pontine reticular form.	0	+	+
Principal trigeminal nu.	+	++	++
Motor trigeminal nu.	0	0	0
Pontine raphe nucleus	0	++	++
Vestibular nuclei	0	+	+
Medulla Oblongata			
Cochlear nuclei		0	0
Spinal trigeminal nu.	+	+++	+
Prepos. hypoglossal nu.	0	0	0
Medullary reticular form.	0	+	+
Inferior olive	0	0	0
Dorsal vagal complex			
Nu. of the solitary tract	++	++	++
Dorsal motor vagal nu.	0	0	0
Area postrema	0	0	0
Motor hypoglossal nu.	0	0	0
Nucleus ambiguus	0	0	0
Medullary raphe nuclei	0	0	0
Cerebellum			
Cortex	++	+	0
Nuclei	0	0	0
Spinal cord	+	++	+

The average number of labeled cells per section in the nucleus is none (0), 1-10 (+), 11-20 (++), or over 20 (+++). Similarly, the density of PTH2R-ir fibers and fiber terminals is represented as none to low (0), moderate (+), high (++), and very high (+++). The table is a slight modification of our previously published table (Faber *et al.*, 2007).