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Tuberoinfundibular Peptide of 39 Residues-Immunoreactive Fibers in the Zona Incerta and the Supraoptic Decussations Terminate in the Neuroendocrine Hypothalamus

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Abstract

Tuberoinfundibular peptide of 39 residues (TIP39) is expressed by neurons in the subparafascicular area, the posterior intralaminar complex of the thalamus and the pontine medial paralemniscal nucleus. TIP39-positive fibers from these areas do not form individual bundles or fascicles, they join other pathways to reach their innervated brain areas. Fibers arise from TIP39 perikarya located in the subparafascicular area and the posterior intralaminar complex of the thalamus could be followed to the hypothalamus. After uni- and bilateral posterolateral surgical deafferentations of the hypothalamus, accumulation of TIP39 immunoreactivity was observed in the fibers caudal to the knife cut, while it disappeared completely rostral to the transection. In serial sections of the forebrain, we could follow TIP39-ir fibers coursing within the zona incerta and the supraoptic decussations. TIP39-positive fibers that join the incerto-hypothalamic pathway reach the medio-dorsal part of the hypothalamus and form moderate to high density networks in the dorsomedial and paraventricular nuclei. The other set of TIP39-positive axons from the subthalamic area join the fibers of the supraoptic decussations and run in an antero-medial direction through the most ventral portion of the hypothalamus up to the retrochiasmatic area, where they crossover. A certain portion of these TIP39-positive fibers terminates in the territories of the arcuate and the medial preoptic nuclei, as well as in the retrochiasmatic area.

Keywords

Paraventricular nucleus; Arcuate nucleus; Dorsomedial nucleus; Preoptic area; Supraoptic decussations; Hypothalamic deafferentation

Introduction

Tuberoinfundibular peptide of 39 residues (TIP39) is produced by neurons in only three cell groups in the brain. These cells have been identified by in situ hybridization histochemistry in rat, mouse, and macaque and by immunohistochemistry in rat and mouse [1-4]. One group of TIP39 neurons is located in the subparafascicular area of the posterior thalamus. This area includes the rostral subparafascicular nucleus (rSPF) and the subparafascicular nucleus (SPF). The second group consists of TIP39 neurons scattered throughout the posterior intralaminar complex of the thalamus (PIC), an area that includes the parvicellular subparafascicular nucleus (SPFp), the posterior intralaminar thalamic nucleus (PIL), and cells in the most caudal and lateral part of the zona incerta (ZI). The third group of TIP39 neurons is found within the medial paralemniscal nucleus (MPL), which is located in the rostral-lateral portion of the pontine tegmentum, just medial to the fibers and the nuclei of the lateral lemniscus [1, 2].

TIP39-immunoreactive (TIP39-ir) fibers are widely distributed in the brain of rats [2] and mice [3] and have nearly identical distributions in these two species [3]. Within the hypothalamus, TIP39-ir fibers form very dense networks in the dorsomedial (DMN), the paraventricular (PVN), and dense to moderate ones in the arcuate, periventricular and medial preoptic nuclei [2].

In the brain, most of the TIP39-ir fibers do not form independent fascicles or fiber tracts. Instead, they may course as solitary pathways or join major tracts constituted by fibers of other neurotransmitters. Very dense accumulations of fibers can be observed along the dorsal and the most ventral parts of the hypothalamus [1, 2]. The dorsal TIP39-positive fibers traverse the zona incerta with a lateral to medial direction while the others run under the surface of the hypothalamus and further lateral, parallel to the optic tract. We examined the hypothalamus in serial sections immunostained for TIP39 from intact rats, and from rats with surgical disconnection of the hypothalamus with knife cuts through more caudally located brain areas, interrupting fibers that arise from TIP39 neurons in the posterior thalamus, the midbrain and the pontine tegmentum. We localized TIP39-ir projections that course among other fibers in the incerto-hypothalamic path, as well as in the supraoptic decussations.

Experimental Procedure

Animals

Adult, male Wistar rats (300–320 g body weight; Gödöllő, Hungary) were housed 3 per cage in the animal care facility of the institution (n = 15). All efforts were made to minimize the number of used animals and their suffering. Rats were kept at a temperature of $22 \pm 1^\circ\text{C}$ with 12–12 h light/dark periods (lights on at 6.00 a.m.), and supplied with dry rat food and drinking water *ad libitum*. Surgical procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the Ethics Committee of the Semmelweis University (Budapest), based on the European Communities Council Directive of 24 November, 1986.

Experimental Surgery

Rats were anaesthetized with i.p. injections of 60 mg/kg sodium pentobarbital. Elongated holes about 2.0 mm diameter were drilled on the skull, 1.5–3.5 mm caudal to the bregma. For caudo-lateral deafferentation of the hypothalamus, a modified Halász-type knife was used [5]. The bayonet-shaped knife with 1.5 mm radius was introduced into the brain at the midline (AP: −1.8; L: 0.0 mm, the cutting edge of the knife was in the coronal plane). Uni- and bilateral transections were performed (6 rats in each group). The knife was lowered until the tip of the knife reached the basal surface of the brain. Then, the knife was moved 1.0 mm caudally in the same position. Then, the knife was rotated 90° in the medial direction to reach the midline (*unilateral cut*). For bilateral cuts, the knife was rotated an additional 90° on the other side of the brain to reach again the coronal plane with the cutting edge of the knife, then moved 1.0 mm rostrally to complete the *bilateral cut*. The cut arched over the medial hypothalamus (Fig. 1), uni- or bilaterally. All of the caudal and the caudolateral neuronal connections of the hypothalamus were transected. All of the animals were allowed to survive for 7 days following transections. Nine of 12 operated rats survived the surgery, and 7 of them were adequate (complete cuts without any gaps all along to the basal surface of the hypothalamus) for further studies, i.e. for immunostainings. Three intact rats were used as controls.

The animals were sacrificed by pentobarbital anaesthesia and perfused transcardially with saline followed by Zamboni fixative solution. The brains were removed and postfixed in the same solution for 4 h, then cryoprotected in 0.1 M phosphate buffer, pH 7.4 (PB) containing 30% sucrose for 24 h.

Immunohistochemistry

Brains were cut in coronal sections of 40 µm thickness on a freezing microtome (Frigomobil, Reichert-Jung). Sections were collected for floating immunostaining using the avidin-biotin-peroxidase method. Sections were rinsed in PB, treated with 0.5% Triton X-100 for 1 h, then the endogenous peroxidase activity was blocked by a 15 min incubation in 3% H₂O₂. After washing in PB, sections were treated with 10% normal goat serum for 1 h, rinsed in PB, and incubated in anti-TIP39 primary antiserum (1:3,000) overnight at 4°C. Next, biotin-labeled secondary antibody, raised in sheep was added (biotinylated anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA), and the sections processed using the ABC technique with a rabbit Vectastain Elite ABC-peroxidase Kit (Vector Laboratories). The tissue bound peroxidase was visualized by fluorescein isothiocyanate (FITC)-tyramide (1:10,000) and H₂O₂ in Tris hydrochloride buffer (0.1 M, pH 8.0) for 10 min. Following the immunohistochemical procedures, sections were mounted, dried overnight, and coverslipped.

Stained sections were examined with Leica DM RX light microscope with fluorescent epi-illumination. Photomicrographs were captured digitally at 1,300 × 1,030 pixel resolution with a Photometrix CoolSnap Fx digital camera. Contrast was adjusted using the “levels” command in Adobe Photoshop.

Results

The knife cuts (Fig. 1) transected all of the fibers that enter the hypothalamus from posterior and lateral directions. Since all of the TIP39 neurons are located posterior to the hypothalamus, the knife cuts resulted in a complete disappearance of TIP39-ir networks of fibers and terminals in the hypothalamus, and in an accumulation of TIP39 immunoreactivity in the transected fibers caudal and lateral to the cut (Fig. 2).

TIP39-ir Fibers in the Zona Incerta and the Incerto-Hypothalamic Pathway

TIP39 neurons are present in the most caudal and ventral portion of the zona incerta. Axons of these neurons join the TIP39-ir axons from the other compartments of the PIC (SPFp and PIL) and the subparafascicular area and course rostrally and slightly medially, within the zona incerta. At the level of the posterior hypothalamus, the fibers reach the rostral part of the zona incerta where the A13 catecholamine cell group is located. From this part of the zona incerta, a track called *incerto-hypothalamic pathway* [6] arises, which enters the hypothalamus and could be further followed to the dorsomedial (DMN) and paraventricular (PVN) nuclei. A fairly high number of TIP39-ir fibers are visible all along in this pathway (Fig. 3).

Hypothalamic cuts transected the incerto-hypothalamic pathway at the levels immediately caudal either the PVN or the DMN. Accumulations of TIP39-ir were seen within fibers on the lateral side of the cut and TIP39-ir disappeared from fibers medial to it (Fig. 2), indicating their course in a latero-medial direction. The dense network of TIP39-ir fibers in the PVN (Fig. 4a) was strongly reduced by unilateral transections, *ipsilateral* to the cut (Fig. 4b). Bilateral knife cut resulted in a *bilateral* disappearance of TIP39 immunoreactivity from the PVN (Fig. 4c). In intact rats, TIP39-ir fibers establish a dense network in the central (compact) part of the dorsomedial nucleus (Fig. 5a), and moderate or slight networks in its ventral and dorsal subdivisions. TIP39-ir disappeared almost completely from these areas in rats with *bilateral* knife cuts (Fig. 5b). It should be noted that TIP39-ir fibers did not disappear from the most caudal portion of the arcuate nucleus in rats if the knife did not reach the basal surface of the hypothalamus (Fig. 5b). In such cases (n = 2), the small gap serves as an open gate for the fibers of the supraoptic decussations (see below).

TIP39-ir Fibers in the Supraoptic Decussations

TIP39-ir fibers join the supraoptic decussations at a location where the fibers enter the subthalamic area and course in a rostral direction between the optic tract and the internal capsule. The TIP39-ir fibers run adjacent to the optic tract (Fig. 6b) and enter the lateral hypothalamus in this position (Fig. 6a). The fibers course gradually toward the midline, just under the surface of the hypothalamus and cross over in the medial retrochiasmatic area, under the third ventricle (Fig. 6c). Here, they may innervate cells in the retrochiasmatic area, and some of the TIP39-ir fibers turn dorsally and enter the periventricular nucleus (Fig. 6c, arrow). Coursing under the surface of the lateral hypothalamic and periventricular areas of the anterior hypothalamus, TIP39-ir fibers enter the ventral portion of the medial preoptic area.

Hypothalamic cuts transected the supraoptic decussations in the lateral retrochiasmatic area (Fig. 2). Strong accumulations of TIP39 immunoreactivity were seen in the transected fibers lateral to the cut, while they disappeared completely in the hypothalamus, i.e. medial to the transection (Fig. 2). In these rats, TIP39-ir fibers disappeared from the arcuate nucleus (Fig. 7), as well as from the medial retrochiasmatic area. Unilateral transection of the suprachiasmatic decussations resulted in a complete disappearance of TIP39-ir fibers in the ipsilateral arcuate and periventricular nuclei without any visible changes on the contralateral side. In contrast, moderate alterations in the density of TIP39-ir network were seen in both sides of the retrochiasmatic area indicating the existence of cross-over of TIP39-ir fibers in this point of the hypothalamus (not shown).

The topography of TIP39-ir fibers within the midbrain and the posterior thalamus projecting to the hypothalamus can be best appreciated in horizontal sections (Fig. 8). TIP39-ir fibers from SPF neurons and those that arise in the SPFp and PIC form a U-shaped path. They course from the SPF laterally and caudally, and pass the SPFp and the caudal part of the PIL

and the zona incerta (Fig. 8d). It should be mentioned that, in this portion of their path, TIP39-ir fibers run together with calcitonin gene-related peptide-immunoreactive fibers [7-9].

Discussion

TIP39-ir significantly decreased but did not disappear completely from the hypothalamic nuclei after bilateral lesions of the SPF [1] indicating the contribution of other TIP39 neurons to the innervation of the hypothalamus. Similarly, lesions of the PIL also resulted in an even more sharp reduction but not complete disappearance of TIP39-ir fibers in the hypothalamic paraventricular, dorsomedial and arcuate nuclei [1]. These findings clearly indicate that both of these cell groups may contribute to the innervation of the endocrine hypothalamus. It appears that TIP39 neurons in the SPF and PIC contribute to a common pathway that, after a short run splits into branches and reach the hypothalamus via either the incerto-hypothalamic pathway or the supraoptic decussations.

The zona incerta begins caudally in the lateral midbrain as a continuation of the midbrain reticular formation, and extends rostrally until approximately the level of the paraventricular nucleus of the hypothalamus [10, 11]. TIP39-ir fibers from neurons of the three nuclei of the PIC join TIP39-ir axons of SPF origin, take a full bend and course rostro-medially in the zona incerta (Fig. 8b). In the rostral part of the zona incerta where catecholamine cells form the A13 cell group (also called the incerto-hypothalamic area by Cheung et al. [12]), fibers of various origins form a loose path called incerto-hypothalamic pathway [6,10, 13, 14]. The TIP39-ir fibers run within this path to the dorsomedial and paraventricular nuclei. Transections of these fibers by hypothalamic cuts completely eliminated TIP39 immunoreactivity in these nuclei (Figs. 4, 5). It is likely that TIP39-ir fibers in the PVN that were eliminated by postero-lateral hypothalamic knife cuts arise in TIP39 neurons both in the SPF and the PIC, since after injections of retrograde tracer in the PVN cells were labeled in the SPF and in the other components of the PIC [15].

At the caudal entrance to the diencephalon, a fairly high number of TIP39-ir axons leave the zona incerta, crossing the internal capsule as they join the supraoptic decussations (Fig. 8a). TIP39-ir fibers remaining in the zona incerta may terminate only in the ipsilateral side of the hypothalamus while some fibers in the supraoptic decussations may cross over in the medial retrochiasmatic area and terminate also in the contralateral side. The supraoptic decussations form a long pathway, which is made up by three bundles of fibers: the dorsal supraoptic decussation, pars dorsalis (Ganser) and pars ventralis (Meynert), and ventral supraoptic decussation (Gudden), as it has been reported and summarized by Gurdjian [16]. There exists a great diversity of opinion concerning the origin, course and destination of the fibers in the supraoptic decussations. However, there is consensus about the well-described segment of this bundle within the diencephalon [17-20] that contains some TIP39-ir fibers. (It is noteworthy that this topographical relationship between the zona incerta and the supraoptic decussations was reported by Ganser [21], more than 125 years ago. Furthermore, Ganser observed that some fibers of the supraoptic decussations turned dorsally in the retrochiasmatic area and ran towards hypothalamic periventricular structures as it is shown for TIP39-ir fibers in Fig. 6c.)

In the diencephalon, TIP39-ir fibers coursed among other fibers in the supraoptic decussations just between the optic tract and the internal capsule (Fig. 6b). After entering into the lateral hypothalamus, TIP39-ir fibers course in the retrochiasmatic area. Here, the knife cuts transected several TIP39-ir fibers that pass the hypothalamus on way to more rostral forebrain areas. It has already been demonstrated that lesions of the SPF or the PIL strongly reduced the number or eliminated TIP39-ir fibers in limbic cortical areas, the

septum and the amygdala [1]. The description of fine topography of these projections is out of the scope of the present study.

There is an increasing amount of evidence that TIP39 influences the activity of hypothalamic neurons. Brain areas where TIP39-ir neurons are located (SPF, PIC) affect the activity of the hypothalamo-pituitary-adrenocortical axis [15, 22-24] and medial preoptic area-related sexual behavior [25]. TIP39 increases the release of corticotropin-, luteotropin- and growth hormone-releasing hormones from in vitro hypothalamic explants, intracerebroventricular injection of TIP39 increases plasma ACTH and luteotropic hormone in vivo in rats [26], and paraventricular nucleus infusion of TIP39 leads to increased plasma corticosterone [27]. Centrally administered TIP39 inhibits arginine vasopressin release in conscious rats [28]. Recent data suggests that TIP39 influences a variety of neuroendocrine cells via modulation of their glutamatergic innervation [27]. These data support a possible regulatory role of TIP39 in the endocrine hypothalamus. Our results that TIP39 neurons in the posterior thalamus reach the endocrine hypothalamus via the zona incerta and the supraoptic decussations suggest that these pathways contribute to the above listed neuroendocrine actions of TIP39. Thus, TIP39 fibers projecting to the paraventricular nucleus may participate in the regulation of corticotropin releasing hormone and vasopressin, TIP39 fibers projecting to the preoptic area might affect the release of gonadotropin releasing hormone whilst TIP39 fibers projecting to the arcuate and periventricular nuclei might affect growth hormone and prolactin secretion by influencing growth hormone releasing hormone, somatostatin, and dopamine neurons, respectively. In addition, information in the present study provides a neuroanatomical background for further functional investigations of the role of this neuropeptide in the brain.

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This article is dedicated to Abel Lajtha. Abel and I met first in 1987 at a meeting in the United States. There, I had the opportunity to introduce myself to him who was a world-famous person, one of the leaders in neurochemistry. Abel was, as usual, very kind. After a long and friendly conversation, we both agreed that there was a need for mapping amino acids and various enzymes in the human, as well as in rat brains. Thus, we decided to do a little work together. We therefore pooled our expertise and entered upon a several-year journey in neuroscience that was both fascinating and somewhat exhausting. We eventually succeeded in publishing 15 articles. The high number of citations of these publications is a clear indication that our expectations were fulfilled. We are still interacting in neuroscience, hopefully we will continue these studies in the future. Abel is a great friend, always kind and very helpful. It is always an intellectual experience for me to meet him either in New York, or Budapest, or somewhere in the globe. I enjoy his great sense of humor and his attitude towards life. He is always positive, optimistic, never complaining—not like me. I feel lucky and privileged to have him for a scientific partner and be a friend of him. With profound appreciation, I wish Abel good health and further creative activity in research. The work was supported by the Hungarian Science Foundation (OTKA): grant No. NK72929 for MP, the Bolyai János Fellowship Grant of the Hungarian Academy of Sciences for AD, and Intramural Research Program of the NIH, National Institute of Mental Health for TBU.

Abbreviations

PB	0.1 M phosphate buffer (pH 7.4)
PIL	Posterior intralaminar thalamic nucleus
PIC	Posterior intralaminar complex of the thalamus
PVN	Paraventricular nucleus
rSPF	Rostral subparafascicular nucleus
SPF	Subparafascicular nucleus
SPFp	Parvicellular subparafascicular nucleus

TIP39 Tuberoinfundibular peptide of 39 residues**References**

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Fig. 1.

Bilateral dorso-lateral hypothalamic deafferentation. Example showing bilateral knife cuts just lateral and dorsal to the hypothalamus. Coronal section through the middle part of the hypothalamus, hematoxylin-eosin staining. The knife cut is indicated by *white arrows*. *HA* habenula, *ME* median eminence, *mt* mamillothalamic tract, *ot* optic tract, *VM* ventromedial nucleus, *3V* third ventricle. *Scale bar*: 1.0 mm

Fig. 2. TIP39-ir following a bilateral knife cut. TIP39 immunoreactivity is seen in fibers lateral to the knife cut while it completely disappeared medial to the cut inside the medial hypothalamus. Accumulations of TIP39 are seen in the transected fibers of the supraoptic decussations (*arrow*) and the incerto-hypothalamic tract (*arrow on the top*). *AH* anterior hypothalamic nucleus, *ot* optic tract, *3V* third ventricle. *Scale bar*: 200 μ m

Fig. 3. Topographical interrelationship between the zona incerta and the paraventricular nucleus. **a** TIP39-ir fibers within the incerto-hypothalamic pathway. The *arrow* shows the direction of the TIP39 fibers in the zona incerta. Coronal section, 1.9 mm caudal to the level of the bregma. **b** The drawing has been taken from the map of Paxinos and Watson [29] with slight modifications. The framed area corresponds to panel **a**. *f* fornix, *mt* mamillothalamic tract, *PV* paraventricular nucleus, *sod* supraoptic decussations, *ZI* zona incerta. *Scale bar*: 200 μ m

Fig. 4. TIP39-ir in the hypothalamic paraventricular nucleus. **a** TIP39-ir network of fibers in the hypothalamic paraventricular nucleus (PV) in an intact rat. **b** Disappearance of TIP39-ir fibers from the PV ipsilateral to a unilateral knife cut (*indicates the side of the cut). No changes in the density of TIP39-ir fibers were apparent in the PV on the intact side. **c** A complete disappearance of TIP39-ir fibers from the PV after a bilateral cut, postero-lateral to the PV (not seen at the level of sections). Coronal sections 1.5 mm caudal to the level of bregma. *AH* anterior hypothalamic nucleus, *f* fornix, *3V* third ventricle. *Scale bar*: 250 μ m

Fig. 5. TIP39-ir fibers in the hypothalamic dorsomedial nucleus. **a** There is a fine network of TIP39-ir fibers in the hypothalamic dorsomedial nucleus in an intact rat. **b** TIP39-ir fibers almost completely disappear from the dorsomedial nucleus following complete bilateral postero-lateral hypothalamic cut. Coronal sections, 3.3 mm caudal to the level of bregma. *f* fornix, *ME* median eminence, *NA* arcuate nucleus, *VM* ventromedial nucleus. *Scale bar:* 200 μ m

Fig. 6. TIP39-ir fibers within the supraoptic decussations in an intact rat. Coronal sections 1.3 (**a, c**) and 2.5 (**b**) mm caudal to the level of bregma. TIP39-ir fibers join the supraoptic decussations and run just on the dorsal surface of the optic tract (**a, b**). **c** TIP39-ir fibers cross over in the medial retrochiasmatic area (*star*), some TIP39-ir fibers are seen in the periventricular area (*arrow*). *AH* anterior hypothalamic nucleus, *ic* internal capsule, *MA* medial amygdaloid nucleus, *ot* optic tract, *sod* supraoptic decussations, *ZI* zona incerta, *3V* third ventricle. *Scale bar*: 200 μ m

Fig. 7. TIP39-ir in the arcuate nucleus. **a** TIP39-ir fibers in the arcuate nucleus in an intact rat. **b** Disappearance of TIP39-ir fibers from the arcuate nucleus after complete bilateral knife cut. Coronal sections 2.7 mm caudal to the level of bregma. *ME* median eminence, *VM* ventromedial nucleus, *3V* third ventricle. *Scale bar*: 200 μ m

Fig. 8.

TIP39-ir fibers-containing pathways in horizontal sections. **a** The drawing, modified from Paxinos and Watson [29], corresponds to the level of the section in **(b)**. *Inset* shows the level of panels **(a)** (2.4 mm) and **(c)** (3.62 mm) dorsal to the level of the interaural line. **b** The topography of TIP39-ir fibers can be recognized in the incerto-hypothalamic pathway and the supraoptic decussations. TIP39-ir neurons are seen in the subparafascicular nucleus and in the posterior intralaminar thalamic nucleus. **c** The drawing corresponding to the level of the section in **(d)**. The TIP39-ir path starts from the SPF, it courses in a lateral and caudal direction (**arrow**) and TIP39-ir fibers from the SPFP and PIL join this path (Panel **b**, **d**). From the posterior intralaminar complex, the fibers bend and course ventrally (as indicated by a dotted line from panel **(d)** to panel **(b)** which shows the level of the forebrain about 1.2 mm more ventrally than the level on the panel **(d)**). After entering the zona incerta, TIP39-ir fibers run rostrally (panel **b**). At the area where the zona incerta has a close contact with the internal capsule a number of TIP39-ir fibers join the supraoptic decussations (**arrow**), while the others course further rostro-medially in the zona incerta (**arrow**). Entering into the hypothalamus, TIP39-ir fibers can be followed to the dorsomedial and the paraventricular nuclei (**arrows**). *Aq* aqueductus cerebri, *DM* hypothalamic dorsomedial nucleus, *fr* fasciculus retroflexus, *ic* internal capsule, *ml* medial lemniscus, *mt* mamillothalamic tract, *ot* optic tract, *PIL* posterior intralaminar thalamic nucleus, *PV* paraventricular nucleus, *SI* substantia innominata, *SN* substantia nigra, *sod* supraoptic decussations, *SPF* subparafascicular nucleus, *SPFP* parvicellular subparafascicular nucleus, *ZI* zona incerta. *Scale bar*: 500 μ m