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Distribution and axonal projections of neurons co-expressing thyrotropin-releasing hormone and urocortin 3 in the rat brain

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Abstract

Thyrotropin-releasing hormone (TRH) decreases food intake when administered intracerebroventricularly or into the ventromedial hypothalamus. However, it is unknown which population of TRH neurons exerts this anorexigenic function. In the rostral perifornical area, the pattern of TRH-expressing neurons is reminiscent of the distribution of neurons expressing urocortin3 (Ucn3) that also inhibits feeding when injected into the hypothalamic ventromedial nucleus (VMN). Since co-localization of TRH and Ucn3 may help to identify feeding-related TRH neurons, the putative co-expression of the two peptides was examined using fluorescent *in situ* hybridization combined with immunofluorescence. Almost all ($95.5 \pm 0.2\%$) Ucn3-immunoreactive neurons in the perifornical area expressed proTRH mRNA, while $49.7 \pm 1.6\%$ Ucn3 neurons were double-labeled in the bed nucleus of the stria terminalis (BNST). Only a few Ucn3/proTRH neurons were found outside these two areas. The distribution of axons containing both Ucn3 and TRH was examined by dual immunofluorescence. Ucn3/TRH fibers heavily innervated the VMN. In addition, high densities of double-labeled axons were observed in the lateral septal nucleus, posterior division of the BNST, medial amygdaloid nucleus, amygdalohippocampal area, and ventral hippocampus, forebrain areas associated with psychological stress and anxiety. We conclude that Ucn3 and TRH are co-expressed in a discrete, continuous population of neurons in the perifornical area and BNST, making Ucn3 a neurochemical marker to define a distinct subset of TRH neurons. The distribution of their axons suggests that Ucn3/TRH neurons may coordinate feeding and behavioural responses to stressful stimuli.

Keywords

TRH; urocortin 3; perifornical; BNST; ventromedial nucleus

Introduction

Thyrotropin-releasing hormone (TRH), a three amino acid amidated peptide, is named after its ability to regulate TSH release from the pituitary, by which it governs the hypothalamic-pituitary-thyroid axis. In the rat brain, hypophysiotropic TRH-synthesizing neurons that project to the median eminence reside in the medial and periventricular parvocellular subdivisions of the paraventricular nucleus (PVN) (Ishikawa et al., 1988; Kawano et al., 1991; Merchenthaler and Liposits, 1994). However, the production of TRH is not restricted to these neurosecretory neurons. TRH is widely expressed in the central nervous system, with an especially high number of TRH expressing neurons in the hypothalamus (Lechan and Segerson, 1989). As a neuropeptide, TRH is secreted from axon terminals and acts on two receptors, TRH-R1 and TRH-R2 that are broadly expressed in the rodent brain (Calza et al., 1992; Heuer et al., 2000; Zabavnik et al., 1993). Non-hypophysiotropic TRH was suggested to regulate a variety of biological functions (for reviews see (Lechan, 1993; Nillni and Sevarino, 1999), including feeding and metabolism. Injection of TRH either into the cerebral ventricles or into the medial hypothalamus potently decreases food intake (Horita, 1998; Suzuki et al., 1982; Vijayan and McCann, 1977; Vogel et al., 1979). In addition, TRH induces hyperglycaemia in rats when it is injected intracerebroventricularly (Ishiguro et al., 1991; Kabayama et al., 1985; Marubashi et al., 1988) or directly into the hypothalamic ventromedial nucleus (VMN) (Shen et al., 1985). The hyperglycemic effect of TRH would be in accordance with its stimulatory action on VMN neurons (Ishibashi et al., 1979; Kow and Pfaff, 1987) that are known to increase blood glucose levels (Tong et al., 2007).

The neuropeptide urocortin 3 (Ucn3) acts on the type 2 corticotropin-releasing factor receptor (CRF-R2) (Lewis et al., 2001), and exerts very similar effects to TRH when injected into the VMN. Ucn3 infusion into the VMN rapidly elevates blood glucose levels and also decreases food intake (Fekete et al., 2007; Li et al., 2007). Interestingly, in addition to the functional similarities between TRH and Ucn3, Ucn3 is expressed in the rostral perifornical area (Lewis et al., 2001; Li et al., 2002) in a pattern reminiscent to the distribution of the perifornical group of TRH neurons (Lechan and Jackson, 1982; Segerson et al., 1987). Moreover, we described in our previous study that perifornical TRH neurons send dense projections to the VMN, lateral septal nucleus, bed nucleus of the stria terminalis (BNST), medial amygdaloid nucleus and amygdalohippocampal area among others (Wittmann et al., 2009), and all of these brain areas are also densely innervated by Ucn3-containing axons (Li et al., 2002).

To investigate the presumed functional/anatomical relationship between TRH and Ucn3, we examined whether these two peptides are expressed by the same neurons. Furthermore, we also determined the axonal projections of the neurons co-synthesizing TRH and Ucn3 by visualizing axons containing both peptides.

Materials and methods

2.1. Animals

Adult male Wistar rats (TOXI-COOP KKT, Budapest, Hungary) weighing 250–350 g were used in this study. Animals were housed under standard conditions (light between 06.00 and 18.00 h, temperature 22 ± 1 °C, rat chow and water available *ad libitum*). All experimental protocols were reviewed and approved by the Animal Welfare Committee at the Institute of Experimental Medicine of the Hungarian Academy of Sciences.

2.2. Combined fluorescent in situ hybridization for proTRH and immunofluorescence for Ucn3

Three animals were anesthetized intraperitoneally with ketamine-xylazine (ketamine: 50 mg/kg body weight; xylazine: 10 mg/kg body weight) and injected intracerebroventricularly with 100 µg colchicine in 5 µl 0.9% saline to increase the number of Ucn3-immunoreactive (IR) perikarya detected by immunocytochemistry. After 20 hours, the animals were deeply anesthetized and perfused transcardially with 20 ml 0.01M phosphate buffered saline (PBS, pH 7.4), followed by 150 ml of 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). The brains were rapidly removed, cut into two blocks caudal to the hypothalamus and cryoprotected in 20% sucrose in PBS at 4°C overnight. Brains were then frozen on dry ice and serial 20 µm thick coronal sections were cut with a freezing microtome (Leica), collected in antifreeze solution (30% ethylene glycol; 25% glycerol; 0.05M PB) and stored at -20°C until used. A one-in-four series of sections from each brain were processed first for *in situ* hybridization. Sections were washed in 2-fold concentration of standard sodium citrate (2×SSC), acetylated with 0.25% acetic anhydride in 0.1M triethanolamine for 10 min and then treated with 50, 70 and 50% acetone, for 5, 10 and 5 min, respectively. After further washes in 2×SSC for 2×5 min, the sections were hybridized with digoxigenin-11-UTP (Roche, Basel, Switzerland)-labeled cRNA probe for rat proTRH. Digoxigenin-labeled antisense proTRH cRNA was synthesized using a 1241 base pair cDNA template corresponding to the coding sequence of proTRH mRNA and portions of its 5' and 3' untranslated sequences (Lechan et al., 1986). The hybridization was performed in polypropylene tubes in hybridization buffer (50% formamide, 2×SSC, 0.25M Tris buffer pH 8.0, Denhardt's solution, 10% dextran sulfate, 0.5% sodium dodecyl sulfate, 265 µg/ml denatured salmon sperm DNA) containing the digoxigenin-labeled probe diluted at 1:75, for 16 h at 52°C. The sections were washed in 1×SSC for 15 min and then treated with RNase A (50 µg/ml; Sigma-Aldrich Co., St. Louis, MO) for 1 h at 37°C. After additional washes in 1×SSC (15 min), 0.5×SSC (15 min) and 0.1×SSC (2×30 min) at 65°C, sections were treated with the mixture of 0.5% Triton X-100 and 0.5% H₂O₂ for 15 min. After washes in PBS, sections were immersed in maleate buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5) for 10 min and in 1% blocking reagent for nucleic acid hybridization (Roche) diluted in maleate buffer. The sections were incubated in Fab fragments of sheep anti-digoxigenin antibody, conjugated with peroxidase (1:100, Roche) in 1% blocking reagent overnight at 4°C. After washes in PBS, the hybridization signal was amplified with biotinylated tyramide for 20 min using the TSA amplification kit (Perkin Elmer Life and Analytical Sciences, Waltham, MA), then sections were incubated in Fluorescein DTAF-conjugated Streptavidin (1:300; Jackson) for 4 h. The sections were rinsed in PBS and incubated for 2 days at 4°C in a rabbit antiserum against human Ucn3 (gift from Dr. Wylie Vale and Dr. Joan Vaughan, The Salk Institute for Biological Studies, La Jolla, CA) at 1:4,000 dilution, preabsorbed with 15 µg/ml rat corticotropin-releasing factor (CRF) (Bachem AG, Bubendorf, Switzerland) for 4 hours. After washing in PBS, sections were incubated in a 1:500 dilution of donkey anti-rabbit IgG conjugated with Alexa Fluor 555 (Invitrogen, Carlsbad, CA) diluted in PBS containing 2% normal horse serum and 0.2% sodium azide (antibody diluent) for 4 h at room temperature. Then, sections were mounted onto glass slides and coverslipped with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA).

2.3 Generation of sheep anti-TRH serum

To study the colocalization of TRH and Ucn3 in axons, we generated an antibody against TRH in sheep using a TRH acrolein bovine serum albumin (BSA) conjugate. The generation of a non-rabbit antiserum was necessitated by the fact that available specific antisera against TRH/proTRH peptides and Ucn3 have been raised in rabbits and it is difficult to obtain reliable double-labeling using two antisera from the same species. The immunogen complex was prepared by mixing 23 mg TRH (Bachem), 24 mg BSA (Sigma) and 15 µl acrolein (Sigma) in 4 ml PBS. The mixture was kept at room temperature overnight. The reaction was stopped

by the addition of 10 mg sodium borohydride. Finally, the conjugate was dialyzed against PBS. For initial immunization, 1200 µg TRH-acrolein-BSA complex in 1 ml PBS was emulsified with an equal volume of Freund's complete adjuvant (Sigma) and injected subcutaneously. Subsequent boosts with Freund's incomplete adjuvant were administered at 28-day intervals. The animal was bled eight days after each immunization and the serum was separated by centrifugation. For this experiment, we used the antiserum from the blood collection after the third immunization (code of the antiserum: 08W2). The antiserum was affinity purified on a column loaded with TRH coupled CNBr-activated Sepharose 4 Fast Flow gel (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK).

2.4. Double immunofluorescence for TRH and Ucn3

Three animals were deeply anesthetized i.p. with ketamine-xylazine and perfused first with 20 ml PBS, followed sequentially by 100 ml of 3% paraformaldehyde/1% acrolein in 0.1M PB and 50 ml of 3% paraformaldehyde in the same buffer. The brains were removed, immersed in 30% sucrose overnight, frozen on dry ice and 25-µm-thick coronal sections were cut on a freezing microtome. Sections were pretreated first with 1% sodium borohydride in distilled water for 30 min, then by 0.5% H₂O₂ and 0.5% Triton X-100 in PBS for 15 min. Non-specific antibody binding was reduced by treatment in 2% normal horse serum in PBS. One-in-four series of sections from each brain were incubated in sheep anti-TRH serum at 1:8,000 dilution for 2 days at 4°C. Following washes in PBS, sections were put into donkey anti-sheep IgG conjugated with Alexa Fluor 555 (Invitrogen) at 1:500 for 2 h at room temperature. Then, sections were incubated in rabbit anti-Ucn3 serum at 1:60,000 dilution, preincubated with 75 µg/ml rat CRF (Bachem), for 2 days at 4°C. Then, sections were immersed in biotinylated donkey anti-rabbit IgG at 1:500 (Jackson) for 2 h, followed by the avidin-biotin-peroxidase complex (ABC Elite Kit, Vector) at 1:1000 for 2 h. After washes in PBS, sections were subjected to biotinylated tyramide amplification for 10 min. After further washes, the sections were incubated in Fluorescein DTAF-conjugated Streptavidin (1:300, Jackson) for 2 h, mounted onto glass slides and coverslipped with Vectashield with DAPI (Vector).

This detection method (Ucn3 with tyramide amplification and Fluorescein DTAF, TRH with Alexa 555-conjugated secunder antibody) resulted in bright, fluorescent signals that were resistant to fading, facilitating the identification and mapping of double-labeled axons using an epifluorescent microscope under a 20× objective. As we previously observed that axons visualized by tyramide amplification may appear thicker than their real size by confocal imaging with standard settings, to obtain high magnification confocal images, both TRH and Ucn3 were detected by direct immunofluorescence in one series of sections from one brain. Sections were incubated for 2 days at 4°C in the mixture of sheep anti-TRH serum at 1:8,000 dilution and rabbit anti-Ucn3 serum at 1:4,000, preabsorbed with 15 µg/ml rat CRF (Bachem). Then sections were immersed in a mixture of donkey anti-rabbit IgG conjugated with Alexa Fluor 488 at 1:250 (Invitrogen) and donkey anti-sheep IgG conjugated with Alexa Fluor 555 (Invitrogen) at 1:500 for 4h at room temperature.

2.5. Antibody characterization

As described by Li et al. (Li et al., 2002), the anti-Ucn3 serum (PBL #6570) was raised in rabbit against synthetic human GlyTyr-Ucn3 conjugated to human α-globulins *via* bisdiazotized benzidine. The specificity of the antiserum was tested for immunohistochemistry by preincubating the antiserum with synthetic human or mouse Ucn3, mouse Ucn2, rat Ucn, or human-rat CRF. Staining for Ucn3 cells and fibers in the brain was completely abolished by preincubation of the antiserum with synthetic mouse or human Ucn3, while Ucn and Ucn2 failed to block staining. CRF was also ineffective in competing Ucn3-specific staining in all areas examined, except the external zone of the median eminence, in which the immunostaining was blocked by preabsorption with a low concentration of CRF. We also observed that the Ucn3

antiserum labels CRF-containing axons in the median eminence, and CRF perikarya in the PVN of colchicine-treated brains. Therefore, we used the Ucn3 antiserum preabsorbed with 15 or 75 µg/ml rat CRF (Bachem) with fluorochrome conjugated secondary antibody or biotinylated tyramide-amplified immunofluorescence, respectively, which abolished all labeling for CRF. The distribution of Ucn3-IR cell bodies was identical with the distribution of Ucn3 mRNA expressing neurons (Lewis et al., 2001; Li et al., 2002).

The generation of the affinity purified sheep antiserum against TRH (code 08W2) is described above. The specificity of the antiserum for immunocytochemistry was tested by preabsorption with TRH (Bachem) at 80 µg/ml concentration, which resulted in the complete loss of axonal and perikaryal staining (Fig. 1A–D). The specificity of the antiserum was further verified by double-labeling of sections with sheep anti-TRH and rabbit anti-TRH no. 31 antiserum (Lechan and Jackson, 1982), that resulted in a complete co-localization of the two signals in axons as well as in cell bodies in colchicine-treated animals (Fig. 1E–H). Our newly generated sheep anti-TRH serum, however, showed a higher sensitivity, as it stained cell bodies of TRH neurons in the PVN of animals without colchicine treatment, while rabbit anti-TRH 31 serum labeled these perikarya only in colchicine-treated animals.

2.6. Image and data analysis

Double fluorescent preparations were examined with a Zeiss AxioImager M1 epifluorescent microscope (Carl Zeiss AG, Göttingen, Germany). To facilitate identification of double-labeled axons and cell bodies, sections were examined under fluorescent illumination through Zeiss Filter Set 23: excitation 475–495 and 540–552 nm, beam splitter 500 and 560 nm, emission 515–530 and 580–630 nm. For unequivocal detection of the signals of individual fluorochromes and for photographing images, the following filter sets were used: for Fluorescein DTAF excitation filter of 450–490 nm, beam splitter of 495 nm, and emission filter of 500–550 nm; for CY3 and Alexa Fluor 555, excitation of 538–562 nm, beam splitter of 570 nm, and emission filter of 570–640 nm. Images were captured either with the Zeiss AxioImager M1 microscope with a 10× objective using an AxioCam MRc 5 digital camera (Zeiss) and AxioVision 4.6 software (Zeiss), or with a Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK). Confocal images were taken using line by line sequential scanning with laser excitation lines 488 nm for Fluorescein DTAF and Alexa Fluor 488, and 543 nm for Alexa Fluor 555; beamsplitter/emission filters, 560/500–530 nm for Fluorescein DTAF and Alexa Fluor 488, and 560–625 nm for Alexa Fluor 555. For 20× and 40× oil lenses, pinhole sizes were set to obtain optical slices of 2 and 1 µm thickness, respectively, and the series of optical sections were recorded with a 2.0 and 1.0 µm Z step. To enhance visibility of double-labeled Ucn3/proTRH perikarya and Ucn3/TRH axons, 2–4 consecutive optical sections were projected into one image with ImageJ image analysis software (public domain at <http://rsb.info.nih.gov/ij/download/src/>). Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA) was used to create composite images and for the modification of brightness and contrast of the images.

Ucn3/proTRH and single-labeled Ucn3 neurons were counted in every fourth 20 µm, serial section from 3 brains, and the mean ± SEM was calculated. Only perikarya with a clearly visible nucleus were counted. Line drawings representing the distribution of double-labeled Ucn3/TRH fibers were made using Corel Draw 11 (Corel Corporation, Ottawa, Canada). For the simple description of the Ucn3/TRH fiber system, projection zones were defined as the followings: *rostral projections*: telencephalic and diencephalic projections rostrally from the level of the anterior pole of the magnocellular division of the PVN; *caudal diencephalic and mesencephalic projections*: posterior from the level of the anterior pole of the magnocellular division of the PVN; *caudal telencephalic projections*: posterior to the substantia innominata.

Results

Co-expression of Ucn3 and proTRH in the perifornical area and in the BNST

To determine the putative co-expression of TRH and Ucn3, we labeled proTRH expressing neurons by fluorescent *in situ* hybridization, and Ucn3-containing neurons by immunofluorescence. Ucn3-IR neurons were found in 4 major cell groups: the perifornical area and BNST, median preoptic nucleus, medial amygdaloid nucleus, and auditory complex of the brainstem. In addition, scattered Ucn3-IR cells were found dorsal to the supraoptic nucleus and periventricularly along the ventral part of the third ventricle. The observed distribution pattern of Ucn3 expressing cells is in agreement with the results of previous studies (Lewis et al., 2001; Li et al., 2002).

In the perifornical area and BNST, two different populations of Ucn3-IR neurons were observed. The distinction between these two groups was made primarily upon the intensity of their Ucn3 immunolabeling, then their distribution and the intensity of proTRH mRNA labeling. The major group of Ucn3 neurons primarily consisted of strongly immunolabeled Ucn3-IR neurons and contained only a few moderately labeled cells. This population of Ucn3-IR neurons was distributed perifornically along the rostrocaudal extent of the PVN (Fig. 2), with the most caudal cells located slightly posterior to the PVN. The cell group continued rostrally in the posterior division of the BNST (Fig. 2A, B), with a few cells extending into the anterior division (divisions by (Ju and Swanson, 1989). The distribution of this cell group is illustrated in a series of images arranged in rostro-caudal order in Fig. 2. A high proportion of these Ucn3-IR neurons expressed proTRH mRNA (Figs. 2, 3A) that was detected in $95.5 \pm 0.2\%$ and $49.7 \pm 1.6\%$ of the cells in the perifornical area and BNST, respectively. Virtually all double-labeled neurons exhibited strong hybridization signal for proTRH mRNA.

The other, relatively minor population of Ucn3-IR neurons consisted of cells labeled faintly by the Ucn3 antiserum (Fig. 3A). Only a few Ucn3 cells in this group were labeled moderately. Rostrally, at the level of the foramen of Monroe, these cells were located immediately ventral and dorsal to the fornix, spatially separated from the few intensely labeled perifornical Ucn3/proTRH-IR neurons present at this level. The posterior part of this cell group was confined to the antero-posterior level of the anterior parvocellular subdivision of the PVN (aPVN), where these cells resided dorsal and just medial to the fornix, or some within the fornix (Fig. 3A). At this level, faintly labeled Ucn3-IR neurons were closely situated to the strongly labeled population of Ucn3 neurons (Fig. 3A). A subpopulation, $19.29 \pm 2.46\%$, of these faint Ucn3-IR cells contained a weak, punctate hybridization signal for proTRH mRNA (Fig. 3A), but none of these neurons were labeled intensely for proTRH mRNA. Cell counts of Ucn3/proTRH and single Ucn3-IR neurons from 3 brains are presented in Table 1. Faint UCN-IR neurons in the perifornical area were not detected without colchicine, whilst the number of intensely stained perifornical Ucn3 neurons was similar in intact (Fig. 5C) and colchicine-treated brains. In colchicine-treated animals, some large neurons within the ventral and lateral parvocellular subdivisions of the PVN were also immunoreactive for Ucn3, but always lacked proTRH mRNA.

The intensely labeled Ucn3/proTRH neurons were not separated strictly from other populations of proTRH-expressing neurons that did not contain Ucn3. There were several single-labeled proTRH mRNA-containing neurons in the vicinity of Ucn3/proTRH neurons both in the BNST and in the rostral part of the perifornical area (Figs. 2A–E, 3A). Most of these single-labeled proTRH mRNA-containing neurons, however, had a less intense TRH hybridization signal compared to the Ucn3/proTRH neurons that had very strong hybridization signals and apparently larger perikarya (Figs. 2A–E, 3A). Perifornical Ucn3/proTRH neurons were also situated very closely to the aPVN either dorsally and/or laterally (Figs. 2B–D, 3A). Some Ucn3/proTRH neurons even intruded into the aPVN, especially at its mid level, intermingling with

the Ucn3 negative proTRH neurons of this subdivision (Figs. 2B–D, 3A). Only 1 or 2 scattered Ucn3/proTRH neurons were found in the medial and periventricular parvocellular subdivisions of the PVN where the hypophysiotropic TRH neurons are located. Caudally located Ucn3/proTRH neurons were partly interspersed with strongly hybridized single-labeled proTRH mRNA-containing neurons (Fig. 2H).

Outside the BNST and perifornical area, only a few neurons were found that co-expressed both Ucn3 and proTRH. ProTRH mRNA was expressed by the few Ucn3 neurons located dorsal to the supraoptic nucleus (Fig. 3B) and in some Ucn3 neurons in the anterodorsal and posterodorsal part of the medial amygdaloid nucleus (Fig. 3C). Double-labeled neurons in these locations were intensely labeled for both Ucn3 immunoreactivity and proTRH mRNA. For the most part, however, Ucn3 neurons in the medial amygdaloid nucleus did not contain proTRH mRNA (Fig. 3C). We did not find any colocalization of proTRH mRNA and Ucn3 in the median preoptic nucleus and in the auditory complex of the brainstem (Fig. 3D, E). Together, double-labeled neurons located dorsal to the supraoptic nucleus and in the medial amygdaloid nucleus comprised only $4.02 \pm 0.16\%$ of all counted Ucn3/proTRH neurons in the brain, and $4.25 \pm 0.14\%$ of all strongly labeled Ucn3/proTRH neurons, if the faintly labeled population of Ucn3/proTRH neurons is not included in the calculation. Table 1 summarizes the cell counts of Ucn3/proTRH cells in the different Ucn3-expressing cell groups.

Distribution of Ucn3/TRH axons in the brain

The distribution of axons containing both Ucn3 and TRH were studied on sections double-immunolabeled for Ucn3- and TRH-immunoreactivity. The distribution of double-labeled fibers is illustrated at different rostrocaudal levels in Fig. 4. Ucn3/TRH fibers were intensely stained for both antigens invariably throughout the brain, suggesting that the axons of the faintly-labeled perifornical Ucn3-IR TRH neurons were not detected by our method.

Rostral projections—Ucn3/TRH fibers densely innervated a relatively large area ventral to the fornix (Figs. 4F, 5C), that included the anterior part of the anterior hypothalamic area, the medial preoptic nucleus, and the interfascicular nucleus of the BNST. This heavy projection continued rostrally and dorsally in the posterior division of the BNST, including its three major nuclei, the principal, transverse and interfascicular nuclei (Dong and Swanson, 2004; Ju and Swanson, 1989) (Figs. 4E, 5D,F). However, the lateral parts of the transverse and interfascicular nuclei received only sparse Ucn3/TRH fibers. The anterior division of the BNST contained only a moderate density of Ucn3/TRH axons. A moderate density of double-labeled fibers was widely distributed throughout the preoptic region. A very high density of Ucn3/TRH fibers was observed in a crescent-shaped area covering the ventral and intermediate parts of the lateral septal nucleus (Figs. 4B,C, 5A). The appearance of double-labeled axons was very characteristic in this region, as Ucn3/TRH axons encompassed the dendrites and cell bodies of lateral septal neurons establishing several consecutive varicosities (Fig. 5B). The medial part of the lateral septal nucleus contained only low to moderate density of Ucn3/TRH axons that often ran dorsally near the midline. These fibers had only few branches and varicosities. Low density of double-labeled fibers was observed in the medial septal nucleus, nuclei of the horizontal and vertical limb of diagonal band, accumbens nucleus, ventral pallidum, and in the rostral part of the lateral hypothalamic area. Scattered double-labeled fibers were observed in the suprachiasmatic nucleus and in the anterior parvocellular subdivision of the PVN.

Caudal telencephalic projections—Moderate density of double-labeled fibers was observed in the substantia innominata, the central amygdaloid nucleus (Figs. 4G,H 6E) and the intercalated amygdaloid nucleus. Moderate density of Ucn3/TRH fibers was observed in the anterodorsal part of the medial amygdaloid nucleus, whereas the posterodorsal and

posteroventral part were very densely innervated by Ucn3/TRH axons (Figs. 4I,J 6A,B). The posterior cortical amygdaloid nuclei and the intraamygdaloid division of the BNST contained moderate densities of Ucn3/TRH axons. The amygdalohippocampal transition area exhibited very high density of branching Ucn3/TRH axons (Figs. 4N–R, 6C,D). High density of Ucn3/TRH axons was found in the rostral part of the ventral hippocampus innervating primarily the CA3 field and the dentate gyrus (Figs. 4N–P, 6F–H). The subiculum also received moderate density of Ucn3/TRH fibers. Most caudally the rostral part of the medial entorhinal cortex contained high density of varicose Ucn3/TRH axons (Figs. 4S, 6I), while low density of double-labeled fibers was observed in the lateral entorhinal cortex. Several passing Ucn3/TRH axons with a few boutons were observed through the stria terminalis and the fimbria of the hippocampus. Ucn3/TRH axons observed in the amygdala, hippocampus and cortex probably travel through these two fiber tracts.

Caudal diencephalic and mesencephalic projections—Double-labeled fibers were distributed widely at low density in the ventral part of the anterior hypothalamic area and the rostral part of the lateral hypothalamic area. Ucn3/TRH axons were densely accumulated in a small area in the antero-lateral part of the tuber cinereum area near the lateral hypothalamic area (Figs. 4H, 5H). Moderate density of Ucn3/TRH axons appeared in the medial subparaventricular zone just rostral to the VMN and in the retrochiasmatic area. This projection continued in the VMN that was inundated by a very high density of Ucn3/TRH axons, particularly its dorsomedial part (Figs. 4H–J, 5E,G). This heavy innervation extended to the zone between the ventromedial and dorsomedial nuclei, to the tuber cinereum area just lateral to the VMN, to the zone between the ventromedial and arcuate nuclei and to the lateral part of the arcuate nucleus. Only scattered Ucn3/TRH axons were observed in the medial part of the arcuate nucleus in its mid level. Moderate density of Ucn3/TRH axons was found in the middle and lateral part of the caudal arcuate nucleus. Some double-labeled fibers were seen in the internal zone of the median eminence, apparently running from one side of the hypothalamus to the other. The rostral part of the dorsomedial nucleus contained moderate to high density of double-labeled fibers primarily in its ventral part, while the density of double labeled fibers decreased toward the caudal end of the nucleus, with only low density of axons in the mid and caudal parts. Moderate density of Ucn3/TRH fibers was present in the ventral premammillary nucleus where the double-labeled axons concentrated in the middle part of the nucleus (Figs. 4K, 5I). Scattered Ucn3/TRH axons were found in the mammillary nuclei, and only occasional fibers were found in the PVN.

In the thalamus, scattered Ucn3/TRH axons were observed in the reuniens thalamic nucleus, anterior part of the paraventricular thalamic nucleus, the lateral and medial habenular nuclei, and in the stria medullaris thalami. In the midbrain scattered Ucn3/TRH fibers were seen in the dorsal periaqueductal grey.

Although we did not examine in detail the contribution of Ucn3/TRH axons to the total Ucn3 and TRH innervation of brain areas, we observed that the Ucn3 innervation of several areas originate exclusively or almost exclusively from Ucn3/TRH neurons. These areas include the lateral septal nucleus, ventromedial nucleus, the posteromedial part of the amygdalohippocampal area, ventral hippocampus and medial entorhinal cortex. Also in the posteroventral part of the medial amygdaloid nucleus Ucn3/TRH axons were spatially separated from the more laterally located single-labeled Ucn3-IR axons. In addition, subregions in the posterior division of the BNST were also innervated exclusively by TRH-containing Ucn3 axons. Although Ucn3/TRH fibers also comprised the majority of TRH axons in several nuclei, Ucn3 negative TRH-IR axons were present at least with low to moderate densities in most of these regions. Notably, however, almost all of the TRH-IR axons in the ventromedial nucleus and the medial entorhinal cortex contained Ucn3.

Discussion

In the present study, we demonstrate that TRH and Ucn3 are co-expressed in neurons of the perifornical area and BNST, defining a neurochemically unique cell population. The axons of Ucn3/TRH neurons were found to be widely distributed in the forebrain innervating several distinct hypothalamic and limbic telencephalic nuclei.

Anatomical findings and relationship with previous studies

In accordance with previous observations, Ucn3 neurons were found to constitute an apparently continuous cell group in the posterior division of the BNST and the rostral perifornical area (Li et al., 2002). Nearly all, more than 95% of the strongly-labeled Ucn3 neurons co-expressed proTRH in the perifornical area, whereas about 50% co-expressed proTRH in the BNST. Since the expression of TRH is more widespread in the BNST and perifornical region and Ucn3/TRH neurons anatomically are not strictly separated from other nearby proTRH expressing cell groups, Ucn3 expression neurochemically, and probably also functionally, defines a subgroup of TRH-synthesizing cells. In particular, Ucn3 expression distinguishes perifornical Ucn3/TRH neurons from the population of TRH neurons in the aPVN that do not express Ucn3. These two cell groups are adjacent to each other and are also interspersed to some extent, as a few Ucn3/TRH neurons were found within the aPVN.

Outside the perifornical area and BNST, we found only a few neurons that co-express Ucn3 and proTRH. These were located dorsal to the supraoptic nucleus and in the anterodorsal or posterodorsal parts of the medial amygdaloid nucleus. It is possible that these “external” Ucn3/TRH neurons are ontogenetically related to the perifornical/BNST population of Ucn3/TRH neurons, since even in the amygdalar population of Ucn3 neurons the Ucn3/TRH neurons were located at the dorsomedial edge of that cell group. Indeed, there is evidence that both the posterior BNST and the anterodorsal and posterodorsal parts of the medial amygdala originate from the anterior entopeduncular area (Garcia-Lopez et al., 2008). The Ucn3/TRH neurons outside the perifornical area and BNST, however, comprised less than 5% of all Ucn3/TRH neurons in the brain, and based on our data, their estimated absolute number is also very low. Thus, the Ucn3/TRH neurons of the perifornical area and BNST are the major source of the Ucn3/TRH axons of the brain and the few double labeled neurons located dorsal to the supraoptic nucleus and in the medial amygdaloid nucleus may have only relatively little contribution to this innervation.

The present results on the distribution of Ucn3/TRH fibers confirm the observations made by earlier studies on the projections of perifornical TRH neurons. By retrograde tract-tracing methods both Ishikawa et al. (Ishikawa et al., 1986) and Merchenthaler (Merchenthaler, 1991) described that perifornical TRH neurons project to the lateral septum. Indirect evidences, based on the enkephalin content of perifornical TRH neurons (Merchenthaler, 1991), also indicated the projections of perifornical TRH neurons to the lateral septum (Sakanaka and Magari, 1989; Szeideemann et al., 1995) and to the BNST (Arluison et al., 1994).

In our previous work we described major projection fields of perifornical TRH neurons by tract-tracing methods, and found moderate to high number of perifornical TRH neurons retrogradely labeled from the VMN, dorsomedial nucleus, ventral premamillary nucleus, medial preoptic area/nucleus, posteromedial BNST, lateral septal nucleus, medial amygdaloid nucleus and the amygdalohippocampal area (Wittmann et al., 2009). Low number of perifornical TRH neurons were also labeled retrogradely from the central amygdaloid nucleus (Wittmann et al., 2009). In addition, anterograde tracer injections covering the rostro-medial part of the perifornical TRH cell group resulted in moderate density of anterogradely labeled proTRH-containing axons in the ventral hippocampus and the substantia innominata

(Wittmann et al., 2009). These data are in accordance with the present findings as all of these regions are innervated by moderate to high density of Ucn3/TRH axons.

Another retrograde tract-tracing study by Cavalcante et al. (Cavalcante et al., 2006) reported that the Ucn3 innervation of the ventral premammillary nucleus originates primarily from the medial amygdaloid nucleus, and only a minor part of the perifornical Ucn3 neurons project to the ventral premammillary nucleus. In our previous study we observed a similar number of perifornical TRH neurons retrogradely labeled from the ventral premammillary nucleus (Wittmann et al., 2009). Our present observation is in agreement with these results, since a moderate density of Ucn3/TRH fibers was found in this nucleus, primarily in its middle part, but the majority of Ucn3 axons in the ventral premammillary nucleus lacked TRH immunoreactivity.

Functional implications

The co-expression of two or more neuropeptides by the same cell group is common in the nervous system. Signaling *via* the release of more neuropeptides may allow a cell group to exert distinct effects on its target neurons under different physiological conditions. The different regulatory effects of Ucn3 and TRH on target neurons seem to be evident since they activate different intracellular signaling mechanisms when bound to their receptors: Ucn3 increases cAMP production through the CRF-R2 (Lewis et al., 2001), whereas TRH-R1 and TRH-R2 are coupled to Gq proteins and signal *via* the phosphoinositide-calcium pathway (Cao et al., 1998; de la Pena et al., 1992; Straub et al., 1990). Convergence of these two pathways has been shown to have synergistic effects in certain cellular processes like generating Ca^{2+} signals (McKinney et al., 1989). How Ucn3 and TRH signaling may interact within specific downstream neurons and influence their activity remains to be resolved. It is also important to note that the fast-acting transmitter of Ucn3/TRH neurons is most likely glutamate, since a subpopulation of Ucn3-TRH neurons express enkephalin (Merchenthaler, 1991) and personal observation) and enkephalin-containing axons that form pericellular baskets in the lateral septum establish asymmetric synapses (Szeideemann et al., 1995). Ucn3/TRH axons, therefore, might be expected to stimulate postsynaptic neurons through synaptic glutamate release as well.

The central anorexigenic effect of TRH is well known, but there is a lack of information about which TRH-expressing cell population(s) decrease food intake. Our findings that Ucn3/TRH axons densely innervate the VMN and constitute almost the entire TRH innervation and the total Ucn3 innervation of this nucleus, strongly suggest that Ucn3/TRH neurons are involved in the regulation of feeding, as both TRH and Ucn3 decrease food intake and increase blood glucose levels when injected to the VMN (Fekete et al., 2007; Li et al., 2007; Shen et al., 1985; Suzuki et al., 1982). Since the electrical stimulation of the VMN inhibits feeding (Beltt and Keesey, 1975; Ruffin and Nicolaidis, 1999) and causes hyperglycaemia (Dubuc et al., 1982), these data indicate that Ucn3/TRH axons exert stimulatory effects on VMN neurons. Reducing food intake probably involves the stimulation of leptin-sensitive neurons located in the dorsomedial part of the VMN (Elmqvist et al., 1997; Elmqvist et al., 1998), a region that also receives the highest density of Ucn3/TRH axons. This cell group of the VMN is critical to maintaining normal energy balance, as the loss of leptin receptor in the VMN causes obesity (Bingham et al., 2008; Dhillon et al., 2006) and increased food intake (Bingham et al., 2008). Importantly, the regulation of VMN neurons by leptin involves altering the sensitivity of VMN neurons to Ucn3, since expression of CRF-R2 mRNA in the VMN increases in response to leptin administration (Nishiyama et al., 1999), while it decreases during starvation when leptin levels fall (Makino et al., 1998).

Another site where Ucn3/TRH neurons may regulate energy balance and decrease feeding is the arcuate nucleus, where Ucn3/TRH axons preferentially innervate the lateral part. This

innervation pattern of the arcuate nucleus raises the possibility that Ucn3/TRH axons innervate the anorexigenic pro-opiomelanocortin (POMC) expressing neurons that reside mostly in the lateral part of the nucleus (Jacobowitz and O'Donohue, 1978; Watson et al., 1978), but not the orexigenic neuropeptide Y cells located in the medial arcuate nucleus (Chronwall et al., 1985). The possibility of the direct stimulation of POMC neurons is also supported by data that Ucn3 injection increases POMC mRNA levels in the arcuate nucleus (Li et al., 2007) where the CRF-R2 is expressed (Chalmers et al., 1995; Van Pett et al., 2000).

Since perifornical Ucn3 mRNA expression is increased in response to restraint stress (Venihaki et al., 2004), and there is a high degree of overlap between the distribution of Ucn3/TRH axons in the brain and the areas expressing c-fos following psychological type of stress (Canteras et al., 1997; Cullinan et al., 1995; Dielenberg et al., 2001; Duncan et al., 1993; Figueiredo et al., 2003; Herman et al., 2005; Kollack-Walker et al., 1997; Liu et al., 2007), Ucn3/TRH neurons may have an important role in the adaptation to stress situations. Within the arcuate and ventromedial nuclei, POMC neurons show c-fos expression to restraint stress and forced swim (Liu et al., 2007), whereas the dorsomedial part of the VMN is typically activated to predator exposure (Canteras et al., 1997; Dielenberg et al., 2001; Figueiredo et al., 2003). These data suggest that anorexia and hyperglycemia induced by Ucn3/TRH axons in these nuclei may represent an important part in the adaptation to stressful stimuli. Indeed, Ucn3/TRH neurons may contribute to the stress-induced reduction in food intake as CRF-R2 deletion abolishes the prolonged phase of restraint-induced anorexia (Tabarin et al., 2007).

Restraint, forced swim and predator exposure also induce a massive appearance of c-fos in the lateral septal nucleus (Cullinan et al., 1995; Duncan et al., 1993; Figueiredo et al., 2003), the densest projection area of Ucn3/TRH neurons. Pharmacological stimulation of CRF-R2 in the lateral septum induces anxiety-like behavior (Bakshi et al., 2007; Henry et al., 2006; Radulovic et al., 1999), and secondary to this, a decrease in food intake (Bakshi et al., 2007). In addition, administration of CRF-R2 antagonists into the lateral septum prevents immobilization-induced anxiety (Radulovic et al., 1999; Todorovic et al., 2007) and also decreases shock-induced freezing behavior (Bakshi et al., 2002; Radulovic et al., 1999). Although another CRF-R2 agonist, urocortin 1, also innervates the lateral septal nucleus (Bittencourt et al., 1999; Kozicz et al., 1998) and serves as a stress-related input from the Edinger-Westphal nucleus (Bittencourt et al., 1999; Gaszner et al., 2004; Kozicz, 2007), urocortin 1 fibers innervate a much smaller area in the lateral septum compared to the distribution field of the Ucn3/TRH axons (Bittencourt et al., 1999). In addition, the two fiber systems show little overlap, with urocortin 1 axons innervating the dorsomedial portions of the intermediate part of the lateral septum (Bittencourt et al., 1999), but not the ventral part of the lateral septum which is the area most affected by stress (Cullinan et al., 1995; Duncan et al., 1993; Figueiredo et al., 2003) and densely innervated by Ucn3/TRH axons. Thus, pharmacological and anatomical data together suggest that the Ucn3/TRH-containing innervation of the lateral septal nucleus contributes to the behavioral adaptation to external stressful stimuli. Anxiety-inducing effects of TRH have not been reported in this area, but intraseptal injection of TRH induces locomotor activation (Sharp et al., 1984), and changes in TRH levels in the lateral septum were reported in alcohol-preferring rats (Morzorati and Kubek, 1993; Nikodemova et al., 1998). It is noteworthy that the appearance of Ucn3/TRH fibers is very characteristic and unique in the lateral septal nucleus: Ucn3/TRH fibers form pericellular baskets around the dendrites and cell bodies of lateral septal neurons, establishing a large number of boutons on the cell surface. These axons forming pericellular baskets exclusively establish asymmetric synaptic specializations with their target cells (Szeideemann et al., 1995). The high number of contacts and the excitatory nature of synapses suggest that Ucn3/TRH axons excite lateral septal neurons and serve as a major driving force for these cells.

Ucn3/TRH neurons probably also contribute to stress-like behavior through their major projection sites in the amygdala/hippocampus region. Besides the lateral septal nucleus, the medial amygdaloid nucleus is the other major brain area that shows intense c-fos induction to a variety of psychological stressors such as restraint, swimming, predator exposure and social interaction (Herman et al., 2005). In addition, lesions or pharmacological inactivation of the medial amygdaloid nucleus reduce defensive responses (Blanchard et al., 2005; Herdade et al., 2006; Li et al., 2004). The ventral hippocampus, another major projection area of Ucn3/TRH neurons, is involved in anxiety-like behavior and lesions of the ventral hippocampus have anxiolytic effects and also reduce defensive behavior in the presence of a predator (Bannerman et al., 2004; Blanchard et al., 2005). The presumed stimulatory actions of Ucn3/TRH axons in these areas seem to promote stress responses and increase anxiety. Ucn3/TRH axons also provide a very dense innervation to the amygdalohippocampal transition area, a poorly investigated brain region which shows intense c-fos expression in hamsters after defeat (Kollack-Walker et al., 1997), and it has a role in fear conditioning in rats (Fujisaki et al., 2004).

Projections of Ucn3/TRH neurons to the BNST may not contribute to the behavioral effects of stress, since CRF-R2 activation in the BNST does not seem to induce anxiety (Sahuque et al., 2006). Within the BNST, Ucn3/TRH axons preferentially innervated the nuclei of the posterior division. This part of the BNST, and primarily the principal nucleus, is involved in the inhibition of the hypothalamic-pituitary-adrenocortical (HPA) axis, as lesions placed in this area augment the HPA axis response to restraint (Choi et al., 2007). These data raise the possibility that the putative stimulation of the posterior BNST division by Ucn3/TRH fibers may restrict the activation of the HPA axis during acute stress. The hypersensitivity of CRF-2R deficient mice to stress would agree with this hypothesis (Bale et al., 2000; Coste et al., 2000).

In conclusion, Ucn3 and TRH are co-expressed in a discrete population of neurons in the perifornical area and BNST. The distribution of Ucn3/TRH axons along with physiological data suggests that Ucn3/TRH neurons may participate coordinately in the regulation of behavioral and metabolic responses to external stressful stimuli, but further studies are required to clarify the roles of this cell population.

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Abbreviations

3V	3rd ventricle
ac	Anterior commissure
aca	Anterior commissure, anterior part
Acb	Accumbens nucleus
ACo	Anterior cortical amygdaloid nucleus
acp	Anterior commissure, posterior part
ADP	Anterodorsal preoptic nucleus
AH	Anterior hypothalamic area

AHi	Amygdalohippocampal area
AHiAL	Amygdalohippocampal area, anterolateral part
AHiPM	Amygdalohippocampal area, posteromedial part
alv	Alveus of the hippocampus
aPVN	Paraventricular hypothalamic nucleus, anterior parvocellular subdivision
Arc	Arcuate nucleus
ArcL	Arcuate nucleus, lateral part
ArcM	Arcuate nucleus, medial part
AVPe	Anteroventral periventricular nucleus
BMA	Basomedial amygdaloid nucleus, anterior part
BNST	Bed nucleus of the stria terminalis
BNSTad	Bed nucleus of the stria terminalis, anterodorsal area
BNSTal	Bed nucleus of the stria terminalis, anterolateral area
BNSTav	Bed nucleus of the stria terminalis, anteroventral area
BNSTif	Bed nucleus of the stria terminalis, interfascicular nucleus
BNSTpr	Bed nucleus of the stria terminalis, principal nucleus
BNSTtr	Bed nucleus of the stria terminalis, transverse nucleus
BNSTia	Bed nucleus of the stria terminalis, intraamygdaloid division
CA1	Field CA1 of hippocampus
CA3	Field CA3 of hippocampus
cc	Corpus callosum
CeA	Central amygdaloid nucleus
CPu	Caudate putamen (striatum)
cst	Commissural stria terminalis
D3V	Dorsal 3rd ventricle
DG	Dentate gyrus
DMN	Dorsomedial hypothalamic nucleus
DPAG	Dorsal periaqueductal gray
f	Fornix
fi	Fimbria of the hippocampus
HDB	Nucleus of the horizontal limb of the diagonal band
I	Intercalated nuclei of the amygdala
ic	Internal capsule
ICjM	Islands of Calleja, major island
IM	Intercalated amygdaloid nucleus, main part
LA	Lateroanterior hypothalamic nucleus

LEnt	Lateral entorhinal cortex
LH	Lateral hypothalamic area
LHb	Lateral habenular nucleus
LGP	Lateral globus pallidus
LPO	Lateral preoptic area
LS	Lateral septal nucleus
LSD	Lateral septal nucleus, dorsal part
LSI	Lateral septal nucleus, intermediate part
LSV	Lateral septal nucleus, ventral part
LV	Lateral ventricle
ME	Median eminence
MeAD	Medial amygdaloid nucleus, anterodorsal part
MEnt	Medial entorhinal cortex
MePD	Medial amygdaloid nucleus, posterodorsal part
MePV	Medial amygdaloid nucleus, posteroventral part
MHb	Medial habenular nucleus
MN	Mammillary nuclei
MPA	Medial preoptic area
MPO	Medial preoptic nucleus
MPOC	Medial preoptic nucleus, central part
MRe	Mammillary recess of the 3rd ventricle
MS	Medial septal nucleus
mt	Mammillothalamic tract
MTu	Medial tuberal nucleus
opt	Optic tract
ox	Optic chiasm
pcf	Precommissural fornix
Pe	Periventricular hypothalamic nucleus
PLCo	Posterolateral cortical amygdaloid nucleus
PMCo	Posteromedial cortical amygdaloid nucleus
PMV	Ventral premammillary nucleus
PV	Paraventricular thalamic nucleus
PVA	Paraventricular thalamic nucleus, anterior part
PVN	Paraventricular hypothalamic nucleus
py	Pyramidal tract
RCh	Retrochiasmatic area

Re	Reuniens thalamic nucleus
S	Subiculum
SCh	Suprachiasmatic nucleus
SFO	Subfornical organ
SI	Substantia innominata
sm	Stria medullaris of the thalamus
SO	Supraoptic nucleus
sox	Supraoptic decussation
SPa	Subparaventricular zone of the hypothalamus
st	Stria terminalis
TC	Tuber cinereum areal VDB, Nucleus of the vertical limb of the diagonal band
VMN	Ventromedial hypothalamic nucleus
VP	Ventral pallidum

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Fig. 1. Specificity controls for sheep anti-TRH serum (no. 08W2) in brain tissues. (A, C) Staining of TRH-IR perikarya in the mid part of the PVN and perifornical area, and TRH-IR axons in the median eminence and arcuate nucleus. (B, D) Preabsorption of the antiserum with 80 $\mu\text{g/ml}$ TRH resulted in the complete loss of the TRH-immunoreactivity. (E–H) Double-labeling the sections with sheep anti-TRH serum and rabbit anti-TRH serum (no. 31) resulted in the labeling of the same neurons (E and F, caudal part of the medial parvocellular subdivision of the PVN and perifornical area), and axons (G and H, median eminence and arcuate nucleus). Scale bar = 200 μm .

Fig. 2.

Fluorescent double-labeled images illustrate the colocalization of Ucn3-immunoreactivity (red) and proTRH mRNA (green) in neurons in the BNST and the perifornical area. Images are arranged in rostro-caudal order; darkfield images of the sections are presented in blue for better visibility of the fornix. The cell bodies of double-labeled Ucn3/proTRH neurons appear in yellow. Both single-labeled Ucn3 and double-labeled Ucn3/proTRH neurons are present in the BNST (on B), whereas almost all intensely labeled Ucn3-IR neurons in the perifornical area contain proTRH mRNA. Dendrites of double-labeled neurons appear in red since proTRH mRNA hybridization signal is fainter in dendrites and is restricted only to the proximal dendritic segments. Note that the most medially located Ucn3/proTRH neurons intrude into

the area of the aPVN and are interspersed with Ucn3 negative proTRH neurons of this subdivision (B–D). Dashed lines indicate the border of the PVN in E–H. Scale bar = 200 μ m.

Fig. 3.

(A) Confocal image showing the colocalisation of proTRH mRNA (green) and Ucn3 (magenta) in the perifornical area at the level of the aPVN. Open arrowheads show the most medial Ucn3/proTRH neurons interspersed with single-labeled proTRH neurons in the aPVN. Arrows indicate faint perifornical Ucn3-IR neurons, white arrowheads point to faint Ucn3-IR neurons that also contain weak proTRH mRNA hybridization signal. (B) Two Ucn3/proTRH neurons dorsal to the supraoptic nucleus. (C) Several single-labeled Ucn3 neurons and two Ucn3/proTRH neurons in the posterodorsal part of the medial amygdaloid nucleus. (D, E) Lack of colocalization between proTRH and Ucn3 in the median preoptic nucleus (D) and the trapezoid body (E). Scale bars = 100 μ m in A; 200 μ m in C (applies to B–E).

Fig. 4. Schematic drawing of sections demonstrate the distribution of Ucn3/TRH-IR axons (x) in the brain.

Fig. 5.

Double immunofluorescent labeling demonstrates the co-localization of Ucn3 (green) and TRH (magenta) in axons in the (A, B) lateral septal nucleus (corresponding drawing: Fig. 4C), (C) interfascicular nucleus of the BNST, anterior hypothalamic area and preoptic nucleus (drawing: Fig. 4F), (D, F) posterior division of the BNST (drawing Fig. 4E), (E, G) ventromedial nucleus (drawing: Fig. 4J), (H) anterolateral part of the tuber cinereum area (drawing Fig. 4H), and (I) the ventral premammillary nucleus (drawing Fig. 4K). Ucn3/TRH-IR axons appear in white due to color mixing. Note the characteristic pericellular baskets formed by Ucn3/TRH axons in the lateral septal nucleus in B (1 μ m thick confocal optical section), and Ucn3/TRH-IR perikarya in C. Images F–I were prepared by projecting three 1 μ m thick confocal optical sections into one plane. Note the several single-labeled Ucn3 axons in the ventral premammillary nucleus in I. Scale bars = 200 μ m in A (applies to A,C,D,E); 50 μ m in B (applies to B, F–I).

Fig. 6.

Double immunofluorescent labeling demonstrates the co-localization of Ucn3 (green) and TRH (magenta) in axons in the (A, B) medial amygdaloid nucleus (corresponding drawing: Fig. 4J), (C, D) amygdalohippocampal area (drawing: Fig. 4P), (E) central amygdaloid nucleus (drawing: Fig. 4H), (F, G) dentate gyrus (drawing: Fig. 4O), (H) CA3 field in the ventral hippocampus (drawing: Fig. 4O), and (I) the medial entorhinal cortex (drawing: Fig. 4S). Images B, D, E, G, were prepared by projecting three 1 μ m thick confocal optical sections into one plane, H was prepared by projecting two 2 μ m thick confocal optical sections into one plane. Scale bars = 200 μ m in A (applies to A,C,F,I); 50 μ m in B (applies to B,D,E,G); and 50 μ m in H.

Table 1
Cell counts of UCN/proTRH and single-labeled Ucn3-IR neurons

Counted in every fourth 20 µm thick section from three colchicine-treated brains

	Ucn3/proTRH	single Ucn3
Bed nucleus of the stria terminalis	91.7±5.5	93.0±7.2
Perifornical region, intensely labeled Ucn3-IR cells	729.7±41.3	34.3±2.9
Perifornical region, weakly labeled Ucn3-IR cells	48.7±9.4	200.7±21.7
Dorsal to the supraoptic nucleus	24.0±5.0	1.7±0.7
Anterodorsal and posterodorsal part of the medial amygdaloid nucleus	12.3±5.8	Numerous, not counted