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Nuclear thimet oligopeptidase is coexpressed with oestrogen receptor alpha in hypothalamic cells and regulated by oestradiol in female mice

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Abstract

Thimet oligopeptidase (EC3.4.24.15, also called EP24.15 and TOP and henceforth referred to as TOP) is a neuropeptidase involved in the regulation of several physiological functions including reproduction. Among its substrates is gonadotropin-releasing hormone (GnRH), an important hypothalamic hormone that regulates the synthesis and release of oestradiol and facilitates female sexual behaviour. Using immunohistochemistry, we found that TOP is expressed in the nucleus of cells throughout the female mouse brain, and in high levels in steroid-sensitive regions of the hypothalamus, which is consistent with previous findings in male rats. Furthermore, dual-label immunofluorescence revealed that TOP and oestrogen receptor α (ER α) coexpress in several reproductively-relevant brain regions, including the medial preoptic area (mPOA), arcuate nucleus (ARC), ventrolateral portion of the ventromedial hypothalamic nucleus (VMNvl), and the midbrain central grey (MCG). Previous studies in rats have shown that oestradiol decreases hypothalamic TOP levels or activity, possibly potentiating GnRH effects. In the present study, analysis by immunohistochemistry revealed that oestradiol decreased TOP immunoreactivity in the VMNvl, while no differences were detected in the mPOA, ARC, or median eminence. Overall, the present findings indicate that TOP is coexpressed with ER α , and oestradiol regulates TOP expression in a brain region-specific manner in female mice, providing neuroanatomical evidence that TOP may function in reproductive physiology and/or behaviour.

Keywords

Thimet oligopeptidase; oestradiol; oestrogen receptor α ; ventromedial hypothalamic nucleus

Introduction

The neuropeptidase thimet oligopeptidase (EC3.4.24.15, also called EP24.15 and TOP and henceforth referred to as TOP) catalyzes the degradation of many peptides involved in essential physiological processes, such as regulation of blood pressure, perception of pain, the immune response, and signaling between brain and endocrine organs (reviewed in 1). Several lines of evidence indicate that TOP also regulates reproduction. For example, studies in humans and rodents demonstrate that TOP is expressed in tissues throughout the body (2), but is most active in endocrine tissues associated with reproduction, including the hypothalamus, pituitary and gonads (e.g. 3–7), suggesting that TOP may function both centrally and peripherally to regulate reproduction. Immunohistochemistry studies in male

rats demonstrate that TOP is found in high concentrations in the hypothalamus and specifically in areas associated with the production and release of gonadotropin-releasing hormone (GnRH) (8, 9). GnRH, which is a substrate for TOP (1), regulates the production and release of gonadal steroid hormones such as testosterone and oestradiol (10). In addition, GnRH has been shown to facilitate sexual behavior in female rodents (11, 12).

Recent studies provide evidence that one of the breakdown products of GnRH degradation by TOP, GnRH₁₋₅, is bioactive and functions in GnRH auto-regulation as well as female sexual behaviour in rodents (reviewed in 13). For example, GnRH promotes lordosis, the hormone-dependent sexually receptive posture the female assumes in order to facilitate intromission by the male (14–16). Oestradiol-primed ovariectomized rodents treated with GnRH display lordosis behaviour (11, 12). However, Wu et al. (17) demonstrated that central administration of GnRH₁₋₅ also induced lordosis in oestradiol-primed ovariectomized rats. In fact, the evidence suggests that GnRH-facilitated lordosis may partially occur via its metabolism to GnRH₁₋₅ by TOP. Central administration of a TOP antibody (the same antibody used in the current study) 60 min prior to GnRH administration in oestradiol-primed ovariectomized rats substantially reduced GnRH-facilitated lordosis, presumably due to the prevention of the metabolism of GnRH to GnRH₁₋₅ by the antibody (17). Consequently, hydrolysis of GnRH by TOP may affect oestradiol-dependent female sexual behaviour.

Additionally, several studies have demonstrated that ovarian steroids control TOP levels and/or activity in brain (e.g. 9, 18, 19). The degree of GnRH degradation at the Tyr5-Gly6 bond in rat brain has been shown to change over the course of the oestrous cycle (20, 21). Furthermore, Pierotti et al. (18) showed that TOP enzymatic activity decreases in the medial preoptic area (mPOA) and median eminence (ME), brain regions involved in GnRH production and release respectively, as well as in the anterior pituitary in ovariectomized rats treated with oestradiol compared to controls. Taken together, these results suggest that ovarian steroid hormones regulate TOP activity in a brain-region-specific manner. In contrast to TOP activity in rats, the enzyme activity in ewe brain remains consistent throughout the estrous cycle (19, 22). It is not known if oestradiol regulates TOP levels in the female mouse brain.

The present study investigated the localization of TOP in the hypothalamus of female mice. This study focused on reproductively-relevant brain regions, including those involved with the synthesis and release of GnRH (mPOA, arcuate nucleus (ARC), and ME), as well as the ventrolateral portion of the ventromedial hypothalamic nucleus (VMNvl) and the midbrain central grey (MCG), two regions important for the control of oestradiol-dependent female reproductive behaviour (16, 23–25). While there are two subtypes of oestrogen receptor (ER) (26), oestrogen regulation of reproductive behaviour appears to be mediated mostly by ER α (27–31). Consequently, the present study used dual-label immunofluorescence to investigate the coexpression of TOP and ER α . In addition, the present study also tests the hypothesis that oestradiol regulates TOP levels in female mouse brain. In particular, Western blot analysis and immunohistochemistry were used to examine whether oestradiol regulates TOP levels in whole hypothalamic samples, and specific reproductively-relevant brain regions, respectively.

Materials and methods

Animals

Female C57BL/6J (19–22 g) were bred at Wellesley College, Wellesley, MA to 7–8 weeks and group-housed in a controlled environment of 25 °C with a reverse light cycle of (12L: 12D). Food and water were provided *ad libitum*. Mice were anaesthetized using a

combination of isoflurane and oxygen administered through a nose cone for bilateral ovariectomy. Animals were given buprenorphine (0.1 mg/kg) as an analgesic immediately following surgery. A one-week recovery period followed surgery to allow clearing of endogenous ovarian hormones. All animal procedures were approved by the Wellesley College Institutional Animal Care and Use Committee (Wellesley, MA, USA).

Western blot analysis of oestradiol regulation of TOP

Twenty-three mice were ovariectomized under isoflurane anaesthesia. The oestradiol group (n=8) was injected seven days following ovariectomy surgery with oestradiol benzoate (EB, s.c., 2 µg in vehicle of 0.1 ml sesame oil, n=16) and injected again two days later with 0.1 ml vehicle. The oestradiol plus progesterone group (n=8) was injected with 2 µg of EB in 0.1 ml vehicle seven days following ovariectomy surgery, and two days later injected with progesterone (100 µg, in 0.1 ml sesame oil containing 5% benzyl alcohol and 15% benzyl benzoate). Control animals (n=7) were injected twice with 0.1 ml of vehicle, first seven days after ovariectomy surgery and again two days later. All animals were euthanized eleven days following ovariectomy surgery by CO₂ overdose followed by decapitation, and hypothalamic brain tissue was rapidly dissected out, snap frozen on dry ice, and stored immediately at -80 °C. Tissue was homogenized using a Teflon homogenizer in 25 mM Tris-HCl, 125 mM KCl, 1 µM ZnCl₂, 10 mM NaF, 1 mM PMSF added fresh, pH 7.8, conductivity 12.0 mS/cm² per gram of tissue. Homogenized tissue was then centrifuged at 13,000 rpm for 30 min at 4 °C, after which the supernatant was removed and stored immediately at -80 °C. Samples were pooled (groups of two, except for one vehicle sample) for a total of 4 hypothalamic samples per treatment. Total protein concentration in each hypothalamic homogenate was determined by Bradford assay. One µg total protein of each hypothalamic homogenate sample was gel electrophoresed on 7.5% polyacrylamide gels containing 1% SDS (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Hypothalamic homogenate samples were analyzed for TOP levels by Western blot as described previously (32). Briefly, membranes were rinsed in 0.1 M TBS with 0.05% Tween-20 (TBS-T), then blocked in TBS-T and 5% nonfat milk at room temperature for 1 hr. Blots were cut at the 50 kDa marker. The upper blot was probed for TOP with a rabbit polyclonal antibody directed against rat TOP at two epitopes KPPAACAGD and LSKGLQVEGC (8) (1:20,000, generously provided by Marc Glucksman, Rosalind Franklin U. of Medicine & Science, Chicago, IL) the lower blot was probed for actin with a monoclonal antibody directed against the N-terminal peptide, Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lysof mouse actin (Chemicon International, Inc., Temecula, CA; 1:75,000) in TBS-T overnight at 4°C. The TOP antibody is highly specific. For example, the antibody does not recognize neurolysin (EC 3.4.24.16), an endopeptidase structurally similar to TOP (8). The following day the membranes were incubated in a horseradish peroxidase-linked donkey anti-rabbit secondary IgG (1:10,000, GE Healthcare UK Limited, Buckinghamshire, UK) or sheep anti-mouse secondary IgG (Amersham Biosciences, Buckinghamshire, UK; 1:10,000) for 1 hr at room temperature, and proteins were detected using an enhanced chemiluminescence kit (ECL; New England Biolabs, Ipswich, MA, USA). Purified recombinant rat TOP protein (a gift from Marc Glucksman) was run as a positive control for each Western blot.

Immunoreactive bands were visualized using a PhosphorImager (STORM Scanner 860, Molecular Dynamics) at an excitation wavelength of 450 nm. Images of each gel were taken and saved as 16-bit grayscale Tiff files and the area of each immunoreactive band was analyzed using ImageQuant software (v. 5.0). TOP levels were normalized to actin, which served as a loading control (33).

Immunohistochemistry

Dual-label immunofluorescence—For immunohistochemical analysis mice were ovariectomized and treated with either EB (2 µg, n=7) or vehicle (n = 7). Forty-eight hours later the mice were injected i.p. with an overdose of sodium pentobarbital (75 mg/kg) and perfused intracardially with saline (0.9%) for 1 minute followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.2) for 8 min at a flow rate of 8 ml/min. Brains were immediately removed, blocked, and incubated in 0.1 M sodium phosphate buffer (pH = 7.2) containing 20% sucrose at 4 °C overnight. Using a freezing rotary microtome, 40 µm coronal sections were collected from the preoptic area through the hypothalamus of each brain and stored in cryoprotectant at −20 °C.

Dual-label immunofluorescence was used to detect cells expressing TOP and ERα. All sections were run through the immunofluorescence protocol simultaneously. Brain sections were incubated in 0.05% TBS buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6) followed by a 90 min incubation in 0.05% TBS buffer containing donkey anti-mouse IgG (0.02 µg/µl) to reduce non-specific background staining. The sections were incubated in 0.05% TBS containing 20% non-immune donkey serum and 1% w/v BSA for 20 minutes to reduce non-specific staining. Sections were then incubated at 4°C overnight in a cocktail containing rabbit anti-TOP (1:5,000) and a rat monoclonal antibody generated against the ligand binding domain of human ERα (H222, 1:50, Santa Cruz Biotechnology) in 0.05 M TBS/NaN₃ buffer (0.02% Triton X-100, 0.02% NaN₃, 0.1% gelatin, pH 7.6) with 1% non-immune donkey serum. The following day, sections were rinsed in 0.05M TBS/NaN₃ buffer, then incubated for 90 min in 0.05M TBS/NaN₃ buffer containing the secondary Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen, A-21207, 1:100) used to visualize TOP and Alexa Fluor 488 donkey anti-rat IgG (A-21208, 1:50) used to visualize ERα. Sections were then rinsed in 0.05M TBS/NaN₃ buffer and then again in 0.05M TBS buffer. Sections were mounted on gelatin-coated slides, left to dry, cleared in distilled water and coverslipped with Gel/Mount mounting medium (Biomedica Foster City, CA).

Controls for dual-label immunohistochemistry included the omission of primary or secondary antibodies. Furthermore, the TOP and H222 primary antibodies were each preadsorbed with recombinant TOP and ERα proteins, respectively.

Immunofluorescence imaging and analysis—Brain sections were matched to the mPOA (plate 31); ARC, VMNvl and ME (plate 46); and the MCG (plate 56) (34). One representative matched section per mouse was used for each brain region. Immunofluorescent images of each brain region for each mouse were captured at 200× magnification using a Leica DM IRBE laser confocal microscope, equipped with Ar/Kr/HeNe lasers that excite at 488 nm (ERα) and 561 nm (TOP), and Leica software was used to capture images. A FieldMaster (Coherent) was used to standardize the output of each laser in mV before use. For each brain region, sections from all animals were imaged on the same day using the same gain and offset settings for each laser. All images with scale bars were saved as JPEG files.

Images were then analyzed using NIS-Elements AR 2.30 software by an investigator blind to treatment group. The scale generated from the confocal was used to convert pixels to microns in NIS element. A rectangular region of interest (ROI) was created for analysis of the mPOA (14566 µm²), ARC (3226 µm²), ME (5105µm²), VMNvl (9966 µm²), and MCG (10210µm²). Cells identified within each ROI were restricted by circularity (0.2–1), area (> 15 µm) and intensity (>90 for TOP-immunoreactive cells and >30 for ERα-immunoreactive cells). For the intensity measure, an intensity value was assigned by the program for each pixel within a cell based on a 0–256 scale for each 8 bit JPEG file. This provided an intensity value for each pixel in each cell. The sum of the intensity values for each cell in

each region (optical integrated density) was then measured for each animal. This provided a measure of the optical integrated density of TOP staining within each region. The intensity cut-off of each protein was determined as the minimum intensity above background staining. The number of cells immunoreactive for both TOP and ER α in each area examined of each brain region of control mice was counted. To determine whether oestradiol alters TOP expression, the number of immunoreactive cells, total area of immunoreactivity (total area of immunoreactive cells (μm^2) within the ROI), and average intensity (average optical integrated density) for TOP within the ROI was measured in each brain area of interest for EB and control mice.

Statistical analyses

JMP 7.0 (SAS Institute Inc., Cary, NC) was used for all statistical analyses. A two-way t-test was used to analyze differences between groups for each experiment. A Shapiro-Wilk W goodness of fit test was used to test for normal distributions and Levene's test was used to test for homogenous variances in all variables. For variables with non-normal distributions and unequal variances, a nonparametric Mann-Whitney U test was used. All means are presented $\pm$ their standard errors, and $p < 0.05$ was considered statistically significant.

Results

Expression of TOP in female hypothalamus

Analysis by Western blot using the TOP polyclonal antibody revealed that TOP protein is expressed in the female mouse hypothalamus at a molecular mass of 78 kDa (Fig. 1), which is consistent with reports from other species (e.g. 8).

Localization of TOP in Female Mouse Hypothalamus

Immunohistochemical staining was used to determine TOP expression in reproductively-relevant brain regions of ovariectomized mice. TOP-immunoreactive (TOP-IR) cells were found throughout the hypothalamus. High levels of TOP-IR cells were observed in the mPOA, ARC, VMNvl, and MCG, while few to no TOP-IR cells were observed in the ME (Fig. 2). We found that TOP was expressed in the nucleus of the cell exclusively (Fig. 3).

Female mouse hypothalamic cells coexpress TOP and ER α

ER α immunoreactive (ER α -IR) cells were observed in the mPOA, ARC, and VMN, which is consistent with previous studies in mice (35) and rats (36). Prior studies using in-situ hybridization histochemistry in female rats demonstrate that oestradiol treatment down-regulates ER in the ARC and VMNvl (37, 38). Furthermore, Blaustein (39) showed that immunolabeling of ER α in the cell nucleus of ovariectomized oestradiol treated rats was reduced with the H222 antibody (generated against the C-terminal ligand binding domain) compared to a polyclonal antibody raised against the N-terminus of the receptor. Consistent with these previous findings, immunofluorescence staining for ER α in the cell nucleus was substantially reduced in EB-treated mice compared to controls in the mPOA, ARC, VMNvl, and MCG (data not shown). Thus, dual-label immunofluorescence was used in control animals only.

The majority of cells containing ER α in the mPOA, ARC and VMNvl express TOP (Table 1). The region with the greatest percentage of ER α -IR cells that also expressed TOP was the mPOA ($95\% \pm 3.9$; see Fig. 4), and the region with the lowest percentage was the VMNvl ($46\% \pm 8.9$; see Fig. 4). In contrast, the greatest percentage of TOP-IR cells that also expressed ER α was in the ARC ($67\% \pm 5.7$), and the region with the lowest percentage was the MCG ($9\% \pm 2.0$). Although, most TOP-IR cells did not express ER α in the MCG, a large percentage of the few ER α -IR cells in this region expressed TOP ($88\% \pm 4.8$).

Oestradiol regulation of TOP in the VMNvl

Western blots were used to determine whether oestradiol regulates TOP expression in whole hypothalamus. Since TOP levels in EB-treated mice were statistically similar to TOP levels in EB + P animals [$t(1\cdot6) = 1.11$, $p = 0.31$], these mice were grouped together. TOP levels in hypothalamic samples of EB-treated and control animals were similar [$t(1\cdot10) = 0.39$, $p = 0.7$] (data not shown).

Immunofluorescence staining of hypothalamic brain sections from ovariectomized mice treated with either EB or an oil control was used to determine whether oestradiol alters TOP expression in reproductively-relevant brain regions. In the VMNvl, EB mice had fewer TOP-IR cells [$t(1\cdot11) = 6.5$, $p < 0.0001$] and a smaller area of TOP immunoreactivity [$t(1\cdot11) = 4.3$, $p = 0.001$] (Figs.5 and 6.). In contrast, no differences in cell number or area of immunoreactivity were found in any other brain region examined [all $p > 0.05$] (Fig. 6). Consistent with data for cell number and area of immunoreactivity, intensity of TOP immunofluorescence [$Z(1\cdot11) = 2.1$, $p = 0.04$] in the VMNvl of EB-treated animals was lower than for control animals; however, no differences in intensity were detected in any other brain region examined [all $p > 0.05$].

Controls for immunohistochemistry

Omission of the primary TOP antibody or preadsorption with recombinant TOP protein resulted in no observable TOP-IR cells, but many detectable ER α -IR cells. Likewise, excluding the H222 antibody and preadsorption with recombinant ER α protein resulted in no ER α -IR cells, but many visible TOP-IR cells. In further confirmation of the specificity of the double-label immunofluorescent technique, intensely labeled TOP-IR cells devoid of ER α -IR cells were observed, as well as ER α -IR cells that lacked TOP-IR.

Discussion

Previous immunohistochemical studies in male rats reveal that TOP is found throughout the brain, and in high levels in the hypothalamus (4· 8). The present study demonstrated that TOP is also expressed throughout the hypothalamus and localized in many steroid-sensitive regions of the female mouse. More specifically, TOP-IR cells were found in a variety of reproductively-relevant brain regions, including the mPOA, ARC, ME, VMNvl, and MCG (Fig. 2). Moreover, we found that cells in the mPOA, ARC, VMNvl, and MCG coexpress TOP and ER α (Table 1 and Fig. 4). These regions regulate hormone-dependent reproductive physiology and sexual behaviour in female mice (40). In addition, given that oestradiol regulation of reproductive physiology and behaviour is predominantly mediated by ER α (28· 41), our present data suggest a role for TOP regulation of hormone-dependent female reproduction.

Several studies have shown that TOP enzymatic activity in brain is attenuated by ovarian steroid hormones (reviewed in 1). The current study found no differences in TOP expression in whole hypothalamic tissue by Western blot; however, this was most likely due to the lack of cellular resolution of this technique that is required to detect effects in specific brain regions. In fact, cellular analysis by immunohistochemistry of specific brain regions revealed that oestradiol influenced TOP expression in a region-specific manner. Oestradiol reduced the number of TOP-IR cells in the VMNvl, but not in the ARC, ME, or MCG of female mice. Furthermore, TOP and ER α coexpressed in these reproductively-relevant brain regions. While both TOP and ER α are expressed in neurons and glia(4· 42· 43); these cell types were not distinguished in the present study. It will be important for future studies to determine the proportion of TOP and ER α coexpression in neuronal versus glial cells.

Although not statistically significant, there appeared to be a decrease in TOP-IR cells in the mPOA of EB-treated mice, which is consistent with previous studies in rats. Pierotti et al. (18) found that oestradiol treatment in ovariectomized rats significantly reduced the catalytic activity of TOP in the mPOA and other specific brain regions. The VMNvl, however, was not examined in the Pierotti study (19). The data presented here are measurements of TOP expression rather than activity and, because expression may not directly indicate activity, it will be important in future studies to investigate the activity of TOP in the VMNvl and other reproductively-relevant brain regions.

Because GnRH is a substrate for TOP, we examined brain regions associated with the production and release of GnRH. In fact, Cleverly et al. (44) present evidence in a recent review demonstrating that TOP is the predominant enzyme metabolizing GnRH. Within the brain, the mPOA contains the most cell bodies of GnRH neurons (45–48), many of which terminate in the ARC and ME (49). ME is the site of GnRH secretion into the hypophysial portal system (47). While the present data suggest that oestradiol does not regulate TOP levels in the mPOA, ARC or ME, it may be that the immunohistochemical technique was not sensitive enough to detect changes in TOP levels in these regions. Alternatively, oestradiol may indirectly affect TOP concentrations after it is secreted into the extracellular space.

The present study found that TOP is localized to the nucleus of cells within the hypothalamus, which is consistent with studies in rat brain (4, 8). Although these studies (4, 8) showed a small amount of cytoplasmic TOP staining in male rat brain, we found TOP exclusively in the nucleus of female mouse brain (Fig. 3). Nuclear localization of TOP is to be expected since the protein contains a nuclear-targeting sequence (50). Although the mechanism is unknown, there is evidence that TOP moves from the nucleus to the cytoplasm, where TOP concentrations in the cytoplasm are negatively correlated with TOP concentrations in the nucleus (42). TOP may hydrolyze GnRH within the cell or in the extracellular space. TOP action within the cell might also be targeted at other cytosolic or nuclear substrates. Within the nucleus, TOP may function to degrade transcription factors and/or interact with proteins in the proteasome (42).

TOP is also thought to hydrolyze peptides in the extracellular space. Once in the cytoplasm, TOP can be secreted to the extracellular space by one of several pathways. While TOP is secreted partially via the classical pathway in AtT-20 cells (51), there is compelling evidence that TOP is secreted via an alternate non-classical pathway in brain (42). Wu et al. (9) have demonstrated that TOP is present in the perivascular space of the ME and hypophysial portal blood, and others have suggested different mechanisms by which TOP may degrade GnRH extracellularly (13). Although not investigated in the current study, TOP secreted from neurons in the ME may affect GnRH concentrations. Therefore, it will be important in the future to investigate the function and activity of extracellular TOP as well as the mechanisms by which TOP is secreted into the extracellular space.

Neuropeptidases play an important role in regulating physiological functions. It has been hypothesized that the GnRH-degrading peptidase TOP mediates reproduction. Immunohistochemical studies from other groups have demonstrated that TOP is expressed in reproductively-relevant brain regions of male rodents (4, 8). Our data provide neuroanatomical evidence that oestradiol reduced both the number of TOP-IR cells and the intensity of TOP immunofluorescence in the VMNvl, a region considered to be an important site for the expression of hormone-dependent female sexual behaviours (15, 16, 52). Our results support other recent findings in rats (17), which suggest that TOP may play a role in regulation of sexual behaviors in female rodents. Future studies using TOP inhibitors

injected into specific brain regions will be useful in elucidating the precise role that TOP plays in reproductive physiology and behaviour.

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Fig. 1. Western blot analysis of TOP in the hypothalamus of female mice. A) TOP expressed in the hypothalamus of mice treated with vehicle (lane 1) or 2 μ g of estradiol benzote (lane 2) at the expected molecular mass of 78 kDa. The position of TOP (78 kDa) is indicated. B) TOP expressed in the hypothalamus of mice treated with vehicle (lane 1). Preadsorption of the TOP antibody with a 20-fold excess of recombinant full-length TOP protein resulted in no visible immunoreactive bands of lanes containing hypothalamic whole cell extracts (lane 2).

Fig. 2. TOP-immunoreactive cells in the A) medial preoptic area, B) arcuate nucleus, C) median eminence (ME), D) ventrolateral portion of the ventromedial nucleus of the hypothalamus, and E) midbrain central grey of ovariectomized control mice. 3V, third ventricle. Images were taken at 200× magnification. Scale bars = 100 μm.

Fig. 3. Nuclear TOP-immunoreactivity in the ventrolateral portion of the ventromedial hypothalamic nucleus (VMNvl) from females treated with A) vehicle or B) estradiol benzoate (2 ug). Images were taken at 400 $\times$ magnification (bars = 20 μ m).

Fig. 4.

Cells in the medial preoptic area (mPOA, panels A–C) and ventrolateral portion of the ventromedial hypothalamic nucleus (VMNvl, panels D–F) coexpress TOP (red) and ER α (green) from a control animal. TOP alone (A and D), ER α alone (B and E) and overlaid image of cells expressing both TOP and ER α (C and F). Cells in the mPOA and VMNvl expressing TOP only (red arrows), ER α only (green arrows) and both TOP and ER α (yellow arrows). Images were taken at 200 $\times$ magnification. Magnification bars = 100 μ m. 3V, third ventricle.

Fig. 5.
TOP-immunoreactive cells in the ventrolateral portion of the ventromedial hypothalamic nucleus (VMNvl) from a female treated with A) vehicle or B) 2 μ g of estradiol benzoate. Images were taken at 200 $\times$ magnification at the same laser power and the same gain and offset on the same day. Magnification bars = 100 μ m.

Fig. 6. TOP-immunoreactivity in the medial preoptic area (mPOA), arcuate nucleus (ARC), median eminence (ME), ventrolateral portion of the ventromedial nucleus of the hypothalamus (VMNvl), and midbrain central grey (MCG) of ovariectomized animals treated with vehicle (control; white bars) or 2 μg of oestradiol benzoate (EB; grey bars). A) Mean number of TOP-immunoreactive cells, B) Area of TOP-immunoreactivity measured as the sum of the number of pixels divided by the area examined, and C) Intensity (optical integrated density) of TOP immunoreactivity * $p < 0.05$.

Table 1

Cells Immunostained for thimet oligopeptidase (TOP), oestrogen receptor α (ER α), or Both in the Medial Preoptic Nucleus (MPO), Arcuate Nucleus (ARC), ventrolateral portion of the Ventral Medial Nucleus of the Hypothalamus (VMHVL), and Periaqueductal Grey (PAG).

Brain region	TOP and ER α coexpression in control mice			
	Total TOP cells	Total ER α cells	% TOP cells expressing ER α	% ER α cells expressing TOP
MPO	183.9 $\pm$ 36.0	86 $\pm$ 21.9	61	95
ARC	68.8 $\pm$ 6.9	68 $\pm$ 7.5	67	67
VMHVL	57.2 $\pm$ 7.3	76 $\pm$ 5.6	59	46
PAG	54.0 $\pm$ 7.5	6 $\pm$ 1.7	9	88

Total cells are presented as means $\pm$ their standard errors