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ORIGINAL ARTICLE

Nuclear Thimet Oligopeptidase is Coexpressed with Oestrogen Receptor α in Hypothalamic Cells and Regulated by Oestradiol in Female Mice

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Thimet oligopeptidase (EC 3.4.24.15; also called EP24.15 and TOP; referred to here as TOP) is a neuropeptidase involved in the regulation of several physiological functions including reproduction. Among its substrates is gonadotrophin-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 (ERα) 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 the effects of GnRH. In the present study, analysis by immunohistochemistry revealed that oestradiol decreased TOP immunoreactivity in the VMNvl, whereas no differences were detected in the mPOA, ARC or median eminence. Overall, the present findings indicate that TOP is coexpressed with ERα, 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.

Key words: thimet oligopeptidase, oestradiol, oestrogen receptor α, ventromedial hypothalamic nucleus.

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The neuropeptidase thimet oligopeptidase (EC 3.4.24.15; also called EP24.15 and TOP; referred to here as TOP) catalyzes the degradation of many peptides involved in essential physiological processes, such as the regulation of blood pressure, perception of pain, the immune response, and signalling between brain and endocrine organs (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 (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 gonadotrophin-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, $GnRH_{1-5}$, is bioactive and functions in GnRH autoregulation, as well as female sexual behaviour in rodents (13). For example, GnRH promotes lordosis, the hormone-dependent sexually receptive posture that the female assumes to facilitate intromission by the male (14–16). Oestradiol-primed ovariectomised rodents treated with GnRH display lordosis behaviour (11, 12). However, Wu *et al.* (17) demonstrated that central administration of $GnRH_{1-5}$ also induced lordosis in oestradiol-primed

ovariectomised rats. Indeed, the evidence suggests that GnRH-facilitated lordosis may partially occur via its metabolism to $GnRH_{1\text{-}5}$ by TOP. Central administration of a TOP antibody (i.e. the same antibody used in the present study) 60 min prior to GnRH administration in oestradiol-primed ovariectomised rats substantially reduced GnRH-facilitated lordosis, presumably as a result of the prevention of the metabolism of GnRH to $GnRH_{1\text{-}5}$ 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 (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 ovariectomised 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. By contrast to TOP activity in rats, the enzyme activity in ewe brain remains consistent throughout the oestrous cycle (19, 22). It is not known whether oestradiol regulates TOP levels in the female mouse brain.

The present study investigated the localisation of TOP in the hypothalamus of female mice. The 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), which are two regions important for the control of oestradiol-dependent female reproductive behaviour (14, 16, 23, 24). Although there are two subtypes of oestrogen receptor (ER) (25), oestrogen regulation of reproductive behaviour appears to be mediated mostly by ERα (26–30). Consequently, the present study used dual-label immunofluorescence to investigate the coexpression of TOP and ERα. In addition, the present study also tested 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, USA) to 7–8 weeks and group-housed in a controlled environment under a reversed 12 : 12 h light/dark cycle at 25 °C. Food and water were provided *ad libitum*. Mice were anaesthetised 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 1-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 ovariectomised under isoflurane anaesthesia. The oestradiol group (n = 8) was injected 7 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 2 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 7 days following ovariectomy surgery, and 2 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, 7 days after ovariectomy surgery and, again, 2 days later. All animals were euthanised 11 days following ovariectomy surgery by CO_2 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 homogenised in 25 mM Tris-HCl, 125 mM KCl, 1 μM $ZnCl_2$, 10 mM NaF, 1 mM phenylmethanesulphonyl fluoride added fresh (pH 7.8), with a conductivity of 12.0 $mS/cm^2/g$ of tissue. Homogenised tissue was then centrifuged at 16 000 ***g*** 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 four hypothalamic samples per treatment. Total protein concentration in each hypothalamic homogenate was determined by the Bradford assay. One microgram total protein of each hypothalamic homogenate sample was gel electrophoresed on 7.5% polyacrylamide gels containing 1% sodium deocyl sulphate (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Hypothalamic homogenate samples were analysed for TOP levels by western blot as described previously (31). Briefly, membranes were rinsed in 0.1 M Tris-buffered saline (TBS) with 0.05% Tween-20 (TBS-T), then blocked in TBS-T and 5% nonfat milk at room temperature for 1 h. 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) (dilution 1 : 20 000; generously provided by M. Glucksman, Rosalind Franklin University of Medicine & Science, Chicago, IL, USA), and 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 (dilution 1 : 75 000; Chemicon International, Inc., Temecula, CA, USA) in TBS-T overnight at 4 °C. The TOP antibody is highly specific. For example, the antibody does not recognise neurolysin (EC 3.4.24.16), an endopeptidase structurally similar to TOP (8). The next day, the membranes were incubated in a horseradish peroxidase-linked donkey anti-rabbit secondary immunoglobulin (IgG) (dilution 1 : 10 000; GE Healthcare UK Limited, Chalfont St Giles, UK) or sheep anti-mouse secondary IgG (dilution 1 : 10 000; Amersham Biosciences, Little Chalfont, UK) for 1 h 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 M. Glucksman) was run as a positive control for each Western blot.

Immunoreactive bands were visualised using a PhosphorImager (STORM Scanner 860; Molecular Dynamics, Sunnyvale, CA, USA) 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 analysed using IMAGEQUANT, version 5.0 (GE Healthcare). TOP levels were normalised to actin, which served as a loading control (32).

Immunohistochemistry

Dual-label immunofluorescence

For immunohistochemical analysis, mice were ovariectomised 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 min 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 nonspecific background staining. The sections were incubated in 0.05% TBS containing 20% non-immune donkey serum and 1% w/v bovine serum albumin for 20 min to reduce nonspecific staining. Sections were then incubated at 4 °C overnight in a cocktail containing rabbit anti-TOP (dilution 1 : 5 000) and a rat monoclonal antibody generated against the ligand binding domain of human ERα (H222, dilution 1 : 50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.05 M TBS/NaN3 buffer (0.02% Triton X-100, 0.02% NaN_3, 0.1% gelatin, pH 7.6) with 1% non-immune donkey serum. The next day, sections were rinsed in 0.05 M TBS/NaN_3 buffer, then incubated for 90 min in 0.05 M TBS/NaN3 buffer containing the secondary Alexa Flour 594 donkey anti-rabbit IgG (A-21207, dilution 1 : 100; Invitrogen, Carlsbad, CA, USA) used to visualise TOP and Alexa Flour 488 donkey anti-rat IgG (A-21208, dilution 1 : 50) used to visualise ERα. Sections were then rinsed in 0.05 M TBS/NaN_3 buffer and then again in 0.05 M TBS buffer. Sections were mounted on gelatin-coated slides, left to dry, cleared in distilled water and coverslipped with Gel/Mount mounting medium (Biomedia Foster City, CA, USA).

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 in Paxinos and Franklin (33) (plate 31); ARC, VMNvl and ME (plate 46); and the MCG (plate 56). 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 (Leica Microsystems, Wetzlar, Germany). A FieldMaster (Coherent, Inc., Santa Clara, CA, USA) was used to standardise 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 analysed using NIS-ELEMENTS AR, version 2.30 (Nikon Instruments, Inc., Melville, NY, USA) by an investigator who was blind to treatment group. The scale generated from the confocal was used to convert pixels to microns in NIS-ELEMENTS. A rectangular region of interest (ROI) was created for analysis of the mPOA (14566 μm^2), ARC (3226 μm^2), ME (5105 μm^2), VMNvl (9966 μm^2) and MCG (10210 μm^2). 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 eight-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 ERα 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 analysis

JMP, version 7.0 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. A two-tailed t-test was used to analyse 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. Data are presented as the mean ± SE. $P < 0.05$ 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 (8).

Localisation of TOP in female mouse hypothalamus

Immunohistochemical staining was used to determine TOP expression in reproductively-relevant brain regions of ovariectomised 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, whereas 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).

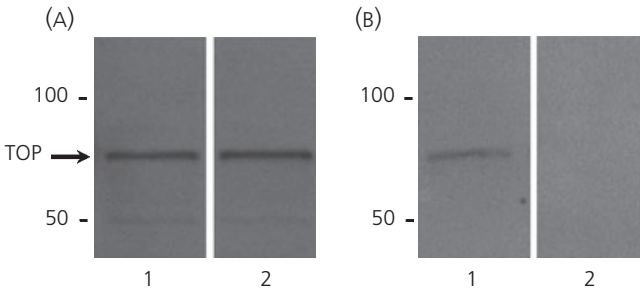


Fig. 1. Western blot analysis of thimet oligopeptidase (TOP) in the hypothalamus of female mice. (A) TOP expressed in the hypothalamus of mice treated with vehicle (lane 1) or 2 μg of oestradiol benzote (lane 2) at the expected molecular mass of 78 kDa. The position of TOP (78 kDa) is indicated. (B)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

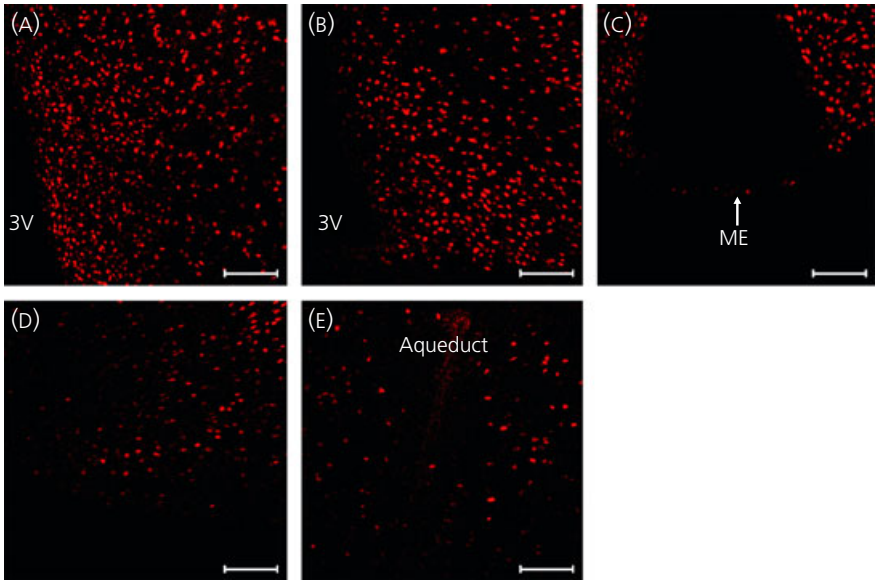


Fig. 2. Thimet oligopeptidase (TOP)-immunoreactive cells in the (A) medial preoptic area, (B) arcuate nucleus, (C) median eminence (ME), (D) ventrolateral portion of the ventromedial nucleus of the hypothalamus and (E) midbrain central grey of ovariectomised control mice. 3V, third ventricle. Images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

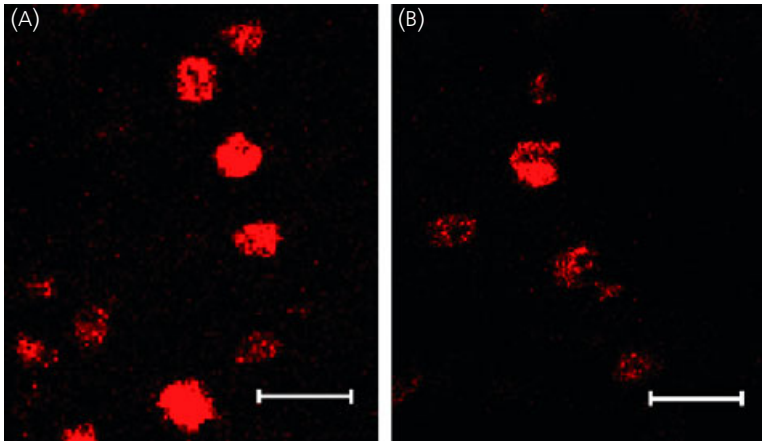


Fig. 3. Nuclear thimet oligopeptidase (TOP)-immunoreactivity in the ventrolateral portion of the ventromedial hypothalamic nucleus from females treated with (A) vehicle or (B) oestradiol benzoate (2 ug). Images were taken at × 400 magnification. Scale bars = 20 μm.

Female mouse hypothalamic cells coexpress TOP and ERα

ERα immunoreactive (ERα-IR) cells were observed in the mPOA, ARC and VMN, which is consistent with previous studies in mice (34) and rats (35). Prior studies using *in situ* hybridisation histochemistry in female rats demonstrate that oestradiol treatment down-regulates ER in the ARC and VMNvl (36, 37). Furthermore, Blaustein (38) showed that immunolabelling of ERα in the cell nucleus of ovariectomised 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 ERα 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 ERα in the mPOA, ARC and VMNvl express TOP (Table 1). The region with the greatest percentage of ERα-IR cells that also expressed TOP was the mPOA (95 ± 3.9%) (Fig. 4), and the region with the lowest percentage was the VMNvl (46 ± 8.9%) (Fig. 4). By contrast, the greatest percentage of TOP-IR cells that also expressed ERα was in the ARC (67 ± 5.7%), and the region with the lowest percentage was the MCG (9 ± 2.0%). Although most TOP-IR cells did not express ERα in the MCG, a large percentage of the few ERα-IR cells in this region expressed TOP (88 ± 4.8%).

Table 1. Cells Immunostained for Thimet Oligopeptidase (TOP), Oestrogen Receptor (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).

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

Total cells are presented as the mean ± SE.

Oestradiol regulation of TOP in the VMNvl

Western blots were used to determine whether oestradiol regulates TOP expression in whole hypothalamus. Because TOP levels in EB-treated mice were statistically similar to TOP levels in EB + progesterone animals ($t_{1,6}$ = 1.11, P = 0.31), these mice were grouped together. TOP levels in hypothalamic samples of EB-treated and control animals were similar ($t_{1,10}$ = 0.39, P = 0.7) (data not shown).

Immunofluorescence staining of hypothalamic brain sections from ovariectomised 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 ($t_{1,11}$ = 6.5, P < 0.0001) and a smaller area of TOP immunoreactivity ($t_{1,11}$ = 4.3, P = 0.001) (Figs 5 and 6). By 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 ($Z_{1,11}$ = 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 pre-adsorption with recombinant TOP protein resulted in no observable TOP-IR cells, but many detectable ERα-IR cells. Similarly, excluding the H222 antibody and pre-adsorption with recombinant ERα protein resulted in no ERα-IR cells, but many visible TOP-IR cells. In further confirmation of the specificity of the double-label immunofluorescent technique, intensely labelled TOP-IR cells devoid of ERα-IR cells were observed, as well as ERα-IR cells that lacked TOP-IR.

Discussion

Previous immunohistochemical studies in male rats have revealed 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 localised 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 ERα (Fig. 4 and Table 1). These regions regulate hormone-dependent reproductive physiology and sexual behaviour in female mice (39). In addition, given that oestradiol regulation of reproductive physiology and behaviour is predominantly mediated by ERα (27, 40), the data obtained in the present study suggest a role for TOP regulation of hormone-dependent female reproduction.

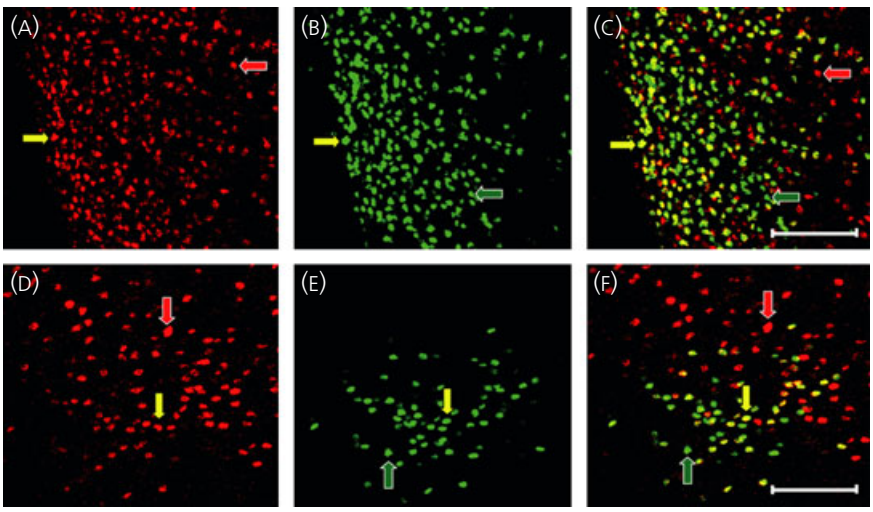


Fig. 4. Cells in the medial preoptic area (mPOA; A–C) and ventrolateral portion of the ventromedial hypothalamic nucleus (VMNvl; D–F) coexpress thimet oligopeptidase (TOP) (red) and oestrogen receptor (ER) α (green) from a control animal. TOP alone (A, D), ERα alone (B, E) and overlaid image of cells expressing both TOP and ERα (C, F).Cells in the mPOA and VMNvl expressing TOP only (red arrows), ERα only (green arrows) and both TOP and ERα (yellow arrows). 3V, third ventricle. Images were taken at × 200 magnification. Scale bars = 100 μm.

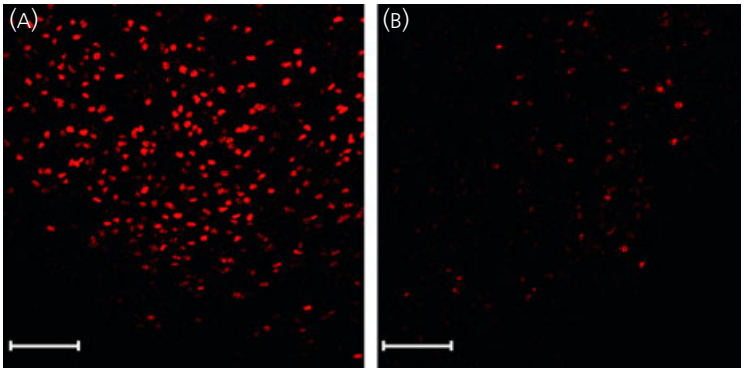
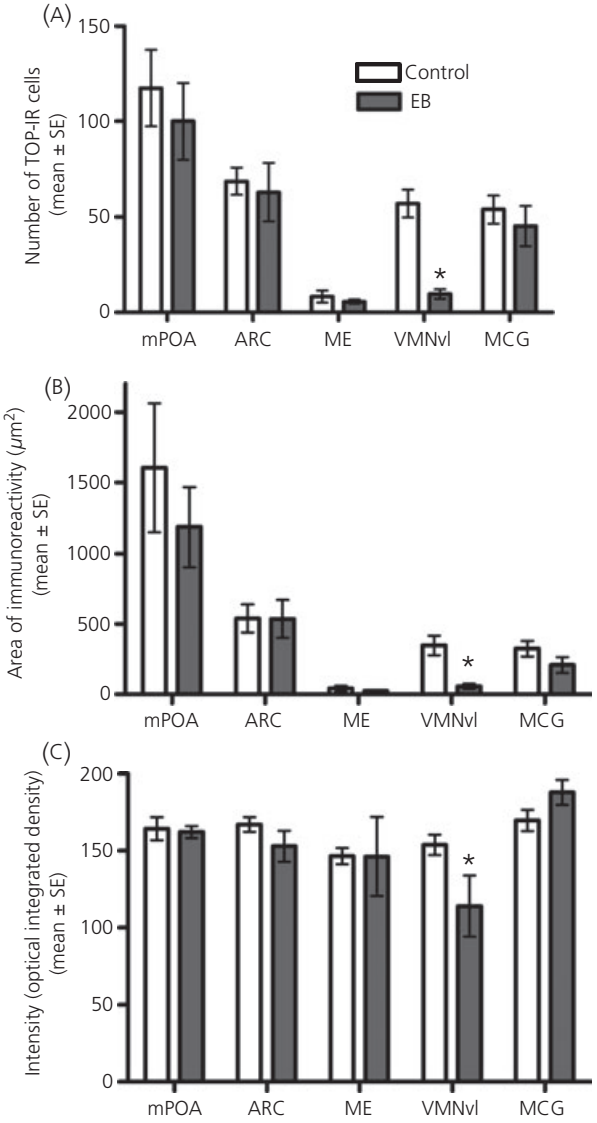


Fig. 5. Thimet oligopeptidase (TOP)-immunoreactive cells in the ventrolateral portion of the ventromedial hypothalamic nucleus from a female treated with (A) vehicle or (B) 2 μg of oestradiol benzoate. Images were taken at × 200 magnification at the same laser power and the same gain and offset on the same day. Scale bars = 100 μm.

Several studies have shown that TOP enzymatic activity in brain is attenuated by ovarian steroid hormones (1). The present study found no differences in TOP expression in whole hypothalamic tissue by western blot; however, this was most likely the result of the lack of cellular resolution of this technique that is required to detect effects in specific brain regions. Indeed, 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 ERα coexpressed in these reproductively-relevant brain regions. Although both TOP and ERα are expressed in neurones and glia (4, 41, 42); these cell types were not distinguished in the present study. It will be important for future studies to determine the proportion of TOP and ERα 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 ovariectomised 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 (18). The data reported in the present study 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. Indeed, Cleverly *et al.* (43) presented evidence in a recent review demonstrating that TOP is the predominant enzyme metabolising GnRH. Within the brain, the mPOA contains the most cell bodies of GnRH neurones (44–47), many of which terminate in the ARC and ME (48). ME is the site of GnRH secretion into the hypophysial portal system

Fig. 6. Thimet oligopeptidase-immunoreactivity (TOP-IR) 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 ovariectomised 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 *$P < 0.05$.

(46). Although 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 localised to the nucleus of cells within the hypothalamus, which is consistent with studies in rat brain (4, 8). Although these studies (4, 8) demonstrated 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 localisation of TOP is to be expected because the protein contains a nuclear-targeting sequence (49). 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 (41). TOP may hydrolyse 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 (41).

TOP is also thought to hydrolyse peptides in the extracellular space. Once in the cytoplasm, TOP can be secreted to the extracellular space by one of several pathways. Although TOP is secreted partially via the classical pathway in AtT-20 cells (50), there is compelling evidence that TOP is secreted via an alternate nonclassical pathway in brain (41). Wu *et al.* (9) have demonstrated that TOP is present in the perivascular space of the ME and hypophysial portal blood, and another study suggested different mechanisms by which TOP may degrade GnRH extracellularly (13). Although not investigated in the present study, TOP secreted from neurones 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 hypothesised 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). The data obtained in the present study provide neuroanatomical evidence indicating 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, 39). These data support other recent findings in rats (17), which suggest that TOP may play a role in the regulation of sexual behaviors in female rodents. Future studies using TOP inhibitors injected into specific brain regions will be useful for elucidating the precise role that TOP plays in reproductive physiology and behaviour.

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