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Dual signal transduction pathways activated by TSH receptors in rat primary tanycyte cultures

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Tanycytes play multiple roles in hypothalamic functions, including sensing peripheral nutrients and metabolic hormones, regulating neurosecretion and mediating seasonal cycles of reproduction and metabolic physiology. This last function reflects the expression of TSH receptors in tanycytes, which detect photoperiod-regulated changes in TSH secretion from the neighbouring pars tuberalis. The present overall aim was to determine the signal transduction pathway by which TSH signals in tanycytes. Expression of the TSH receptor in tanycytes of 10-day-old Sprague Dawley rats was observed by in situ hybridisation. Primary ependymal cell cultures prepared from 10-day-old rats were found by immunohistochemistry to express vimentin but not GFAP and by PCR to express mRNA for Dio2, Gpr50, Darpp-32 and TSH receptors that are characteristic of tanycytes. Treatment of primary tanycyte/ependymal cultures with TSH (100 IU/l) increased cAMP as assessed by ELISA and induced a cAMP-independent increase in the phosphorylation of ERK1/2 as assessed by western blot analysis. Furthermore, TSH (100 IU/l) stimulated a 2.17-fold increase in Dio2 mRNA expression. We conclude that TSH signal transduction in cultured tanycytes signals via Gas to increase cAMP and via an alternative G protein to increase phosphorylation of ERK1/2.

Introduction

The interface between the third ventricle, hypothalamic neuropil and median eminence is composed of cuboidal ependymal cells and specialised ependymoglia cells called tanycytes. These cells have a distinct morphology. They interface with the cerebrospinal fluid in the ventricle and send a single process deep inside the neuropil of the hypothalamus towards the arcuate nucleus (ARC), the ventromedial nucleus (VMH) and the dorsomedial nucleus (DMH). Ventrally, tanycyte processes are localised in the median eminence, where they appose peptidergic terminals and portal capillaries (Rodriguez et al. 2005). Tanycytes sense biologically active hormones and metabolites from the ventricle and portal blood vessels, and they connect the ventricle and neighbouring pars tuberalis via networks of cisterna (Guerra et al. 2010, Frayling et al. 2011, Bolborea & Dale 2013, Balland et al. 2014). They express receptors and/or transport proteins for a wide variety of known and unknown biologically active compounds (Graham et al. 2003, Rodriguez et al. 2005, Barrett et al. 2007, Coppola et al. 2007, Cottrell et al. 2009, Nilaweera et al. 2011, Shearer et al. 2012, Dardente et al. 2014). Notably, tanycytes express type 2 deiodinase (Dio2) at very high levels. Dio2 is an enzyme that converts the weakly active form of thyroid hormone L-thyroxine (3,3',5,5'-tetraiodo-L-thyronine (T4)) to the biologically active form triiodothyronine (T3) (3,5,3'-triiodo-L-thyronine, or T3). This enzyme is important in diverse physiological responses, including fasting and non-thyroid illness caused by bacterial infections, and in both

cases, tanycytic Dio2 expression and T3 production are increased (Klosen et al. 2002, Sanchez et al. 2010). The importance of tanycytes as a source of T3 is also evident in seasonal mammals: photoperiod-regulated T3 availability determines seasonal physiology and behaviour (Barrett et al. 2007, Dardente et al. 2014). Expression of Dio2 is stimulated in tanycytes in a variety of situations, including starvation, which is caused by an unknown mechanism (Coppola et al. 2007), inflammatory conditions, which are caused by an NFkB-dependent signalling mechanism (de Vries et al. 2014, Wittmann et al. 2014), and long-day photoperiods, which are caused by thyroid stimulating hormone (TSH) of pars tuberalis origin (Hanon et al. 2008, Nakao et al. 2008, Ono et al. 2008, Helfer et al. 2013, Herwig et al. 2013, Klosen et al. 2013). TSH receptors are highly localised within the ventral region of the ependyma lining the third ventricle, a region that is composed mainly of tanycytes (Ross et al. 2011, Herwig et al. 2013). I.c.v. administration of TSH elicits a robust increase in Dio2 expression in the ependymal wall (Helfer et al. 2013, Yoshimura 2013), and consequently increases local thyroid hormone availability. The activation of adenylate cyclase is a signal transduction pathway commonly associated with TSH receptor activation, but TSH receptors are known to couple with a diverse range of G proteins to activate several different pathways that potentially have multiple downstream consequences for responses to TSH (Laurent et al. 1987, Allgeier et al. 1994, Kursawe & Paschke 2007, Buch et al. 2008). Our objective was to determine the pathways by which TSH signals within the cells of the ependymal wall, because these may have further consequences for the function of tanycytes and their regulation of the surrounding hypothalamus. In the present study, we used dissociated primary hypothalamic ependymal cell cultures from 10-day-old rats to determine the intracellular signalling pathway utilised by the TSH receptor in these cells.

Materials and methods

Animals

Sprague Dawley rats and their litters were kept under controlled light/dark cycle (12 h light:12 h darkness cycle) and constant temperature ($20\pm 2^{\circ}\text{C}$) and humidity ($55\pm 10\%$) in standard rat cages (type RC2/f). Food (CRM (P) rat and mouse breeder and grower, standard pelleted diet, Special Diet Services, Witham, Essex, UK) and water were provided ad libitum. We used 10-day-old neonates of both sexes. Experimental procedures were approved by the Rowett Institute Ethics Committee, and animals were euthanised under the Schedule 1 of the Animals (Scientific Procedures) Act of 1986 (UK).

Primary cell culture

Brains were collected and micro-dissected in ice-cold Dulbecco's PBS solution (Sigma–Aldrich) using a binocular magnifying microscope. The preparation of primary cell cultures was achieved using the protocol previously described by Prevot et al. (2003) and others (McCarthy & de Vellis 1980, Ma et al. 1994, de Serano et al. 2004). Briefly, after clearing the meninges and blood vessels, the median eminence and the floor of the third ventricle were micro-dissected. Micro-dissected tissues from a minimum of 20 rat pups were pooled in ice-cold DMEM (Sigma Aldrich), then centrifuged for 1 min at 1500 g, and the medium was exchanged for fresh DMEM. Explant tissue was then scraped through a 20 mm mesh over a Petri dish containing DMEM (Sefar, Lancashire, UK). The dissociated tissue was transferred to a centrifuge tube and spun for 5 min at 1500 g. The supernatant was discarded, and the resulting pellet was resuspended by trituration with a Pasteur pipette in 5 ml DMEM supplemented with 10% foetal bovine serum and antibiotic/antimycotics. The cell suspension was transferred to a 25 cm culture flask (Corning Costar, Loughborough, Leicestershire, UK or Sigma–Aldrich) with an additional 5 ml of supplemented medium. Cultures were incubated in a humid atmosphere of 5% CO₂: 95% air at 37 °C. Once cells had adhered (after 3–4 days), the medium was changed every 3–4 days until the cells were confluent. A similar procedure was used with tissue from the cortex to generate a control culture of cortical glia.

Immunohistochemistry

Immediately after removal, brains were immersed in a solution of 4% paraformaldehyde in 95 mM phosphate buffer (20 mM NaH₂PO₄, 75 mM Na₂HPO₄, pH 7.4) and fixed for 24 h with gentle agitation. The brains were cryoprotected by immersion in a gradient of 10, 20 and finally 30% sucrose in PBS. Fourteen micron sections were then cut on a cryostat and immunostained. Briefly, slides containing cut brain sections were rinsed with two washes in 1xPBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and a final wash in 1xPBS + 0.3% Triton X-100 (Sigma–Aldrich) to facilitate cell permeability. This was followed by a 60 min incubation in a blocking buffer that contained 1xPBS + 0.3% Triton X-100 + 5% BSA (Sigma–Aldrich). The primary antibodies were incubated overnight at 4 °C: vimentin (Clone V9, Sigma–Aldrich; dilution 1:1000) and GFAP (ab4674, Abcam; dilution 1:1000). The following day, slides were washed in 1xPBS and then incubated with the secondary antibodies, respectively raised against the appropriate species and coupled to fluorescein (Vector Labs, Peterborough, Cambridgeshire, UK) at 1:1000 or Northern Lights637 (R&D Systems, Abingdon, Oxford, UK) at 1:2000 dilutions.

In situ hybridisation

Brains were extracted in the same manner as they were for cell cultures and immunohistochemistry but rapidly frozen on dry ice. In situ hybridisation was performed on 14 mm frozen sections of 10-day-old rat brain sections as described previously (Shearer et al. 2012). We used a ³⁵S-labelled anti-sense riboprobe for the TSH receptor as described previously (Herwig et al. 2013). Following hybridisation, sections were apposed to film for 7 days.

cAMP assay

After cells reached confluence in the original flask, cultures were plated at a cell density of 100 000 cells/well in a 24-well plate. After a 48 h recovery period, followed by overnight serum deprivation (16 h), cells were rinsed with DMEM three times. Cells were then immediately treated with the appropriate conditions – DMEM only for controls, DMEM containing bovine TSH at 1, 10 and 100 IU/l or forskolin at 10 mM for 60 min – before the media was removed for the cAMP assay. A colorimetric ELISA (Arbor Assay, Ann Arbor, MI, USA) was used to detect egressed cAMP levels. The optical density was measured on a plate reader at 450 nm. Adenylate cyclase activation by TSH was performed in triplicate and in two independent experiments.

MAPK assay

Primary tanycyte cell cultures were plated in a 60 mm petri dish at a density of 300 000 cells/dish and left to adhere and divide for 48 h. The cell cultures were then serum deprived for 16 h (overnight). The following morning, cells were rinsed three times with DMEM at 37 °C. Cells were then incubated for 5 min at 37 °C with DMEM only, bovine TSH in DMEM (1, 10 and 100 IU/l) or forskolin (10 mM). Where cells were pretreated with cholera toxin (Sigma–Aldrich) to inactivate adenylate cyclase, the toxin was added to the DMEM during the overnight serum deprivation at 200 ng/ml. Each treatment was performed in duplicate or triplicate, and the assay was performed twice. After a 5 min treatment, the dishes were immediately placed on ice, the medium was removed and the cells were washed twice with an ice-cold 10 mM HEPES 150 mM NaCl (HN) solution. Cells were scraped off with 100 µl HN solution and immediately transferred to a microfuge tube with 100 µl of 2x Laemmli gel loading buffer (4% SDS, 20% glycerol, 0.125 M Tris–HCl, pH 6.8, 10% βmercaptoethanol). The cells were then sonicated for 6x10 s bursts at a 5 micron amplitude setting (MSE Soniprep 150; MSE, London, UK) to disrupt cell structure and shear DNA. This was followed by 5 min at 95–100 °C to denature the proteins. Twenty microliters lysate was loaded onto a 7x8cm 10% SDS polyacrylamide gel and electrophoresed at 150 V for 2–3 h to

separate the protein components by molecular mass. Proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) using a wet transfer apparatus (Bio-Rad Laboratories). Protein detection was accomplished using a standard western blot protocol. Briefly, membranes were blocked with 5% non-fat dry milk with 0.1% Tween-20 in 1xTBS (20 mM Tris-HCl, 137mM NaCl, pH7.6). Antibodies were incubated overnight at 4 °C in 1xTBS containing 5% BSA with 0.1% Tween-20. The antibodies used were anti-phospho-ERK1/2 (New England Biolabs, Hitchin, Hertfordshire, UK; 1:1000 dilution), an anti-ERK1/2 (New England Biolabs; 1:1000 dilution) and an anti-vimentin (Sigma-Aldrich; 1:5000 dilution). Following washes in 1xTBS with 0.1% Tween-20, an appropriate secondary antibody at a 1:2000 dilution that was linked to HRP (New England Biolabs) was used the next day on the PVDF membrane for 1 h. Proteins were visualised by chemiluminescence using a Pierce Supersignal West Picochemiluminescent substrate (Scientific Laboratory Supplies, East Riding, Yorkshire, UK).

RT-PCR and qRT-PCR

Brain explants were dissected as described in the previous section for primary cell cultures. Total RNA was extracted from brain tissue or primary cell cultures using a QIA shredder (Qiagen) and an RNeasy Mini Kit (Qiagen) on columns and with a DNase 1 (Promega) treatment. Yield and purity of the RNA was quantified with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, MA, USA) and Bioanalyzer 2100 (Agilent Technologies, Cheshire, UK). One microgram of total RNA was reverse transcribed using Superscript II (Invitrogen). Partial sequences of Dio2, TSHr, Gpr50, Vimentin, Darpp32, G3pdh and TSHb were then amplified from the brain explants cDNA templates using a HotStart GoTaq kit (Promega, Southampton, Hampshire, UK) and following primers based on rat and mouse sequences in the Genbank database (f, forward primer; r, reverse primer): Dio2 (NM_031720)-f: CTCTTCCTGGCGCTCTATGACTCG, Dio2-r: TCCTCTTGGTTCCGGTGCTT (494 bp); TSHr (NM_012888)-f: TCCAGGG MCTATGCAATGAAC, TSHr-r: CAGCCCGAGTGAGGTGGAGGA (312 bp); Gpr50 (NM_001191915)-f: AAGCTCCGAAATTCTGGCAACA, Gpr50-r: ATGAGAGGGAGG ACGAAGTGGATG, (434 bp); Vimentin (NM_031140)-f: AGAACACCCGCACCAACGAGAAGG, Vimentin-r: ACGC AGGGCAGCRGTGAGGTC (521 bp); Darpp32 (NM_138521)-f: CTTCTGGGAGCTKGGGTAYC, Darpp32-r: AGGGAAAGGCATTGGGGACTCTG (461 bp); G3pdh (AF106860)-f: ACCACAGTCCATGCCATCAC, Darpp32-r: TCCACCACCCTGTTGCTGTA (451 bp); TSH β (NM_013116)-f: CCGAAGGGTATAAAATGAACAGAG, TSHb-r: ACCAGATTGCATTGCCATTACAGT (505 bp). The cycling conditions used were 60 °C for the annealing temperature for Dio2, Vimentin, Darpp32, G3pdh and TSHb and 58 °C for Gpr50 and TSHr for 40 cycles. The resulting PCR

amplification products were separated through 2.0% (w/v) agarose gels (Melford Laboratories, Ipswich, Suffolk, UK) and stained with SYBRSafe DNA gel stain (Invitrogen, Life Technologies, Paisley, Renfrewshire, UK). To control for the product size, the samples were compared to a 100 bp size marker (Hyperladder IV, Bioline, London, UK). For quantitative real-time PCR (qPCR) analysis, primary cultured cells were plated in 60mm petri dishes at a density of 300 000 cells/dish. Forty-eight hours later, cells were serum starved overnight and then treated either with DMEM or DMEM containing bovine TSH (100 IU/l) for 7 h at 37 °C. Total RNA was extracted and reverse transcribed as described in the previous paragraph. Five nanograms of cDNA were used as template in the qPCR using a QuantiFast SYBRGreen PCR kit (Qiagen, Venlo, The Netherlands) on a Thermal Cycler 7500 Fast RealTime PCR System (Applied Biosystems, Life Technologies). We used Qiagen-validated primers for b-Actin (Quanti Tect Primer Assay, Rn_Actb_1_SG) and Dio2 (Rn_Dio2_2_SG). Each PCR procedure included a negative control reaction without template, and each sample was run in triplicate with the experiment being performed twice. The reaction conditions were as follows: amplification for 5 min at 95 °C, 40 cycle of 10 s at 95 °C, 30 s at 60 °C and dissociation curve analysis for 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. The b-actin housekeeping gene was used as a reference for the relative quantification of Dio2 calculated based on the 2KDCT method.

Results

Expression of the TSH receptor in 10-day-old rat hypothalamus

In situ hybridisation confirmed that TSH receptor expression in the 10-day-old rat brains was localised in the hypothalamus exclusively to the ependymal cell layer lining the wall of the third ventricle (Fig. 1A and A').

Primary tanycyte cultures

Immunocytochemical staining of the 10-day-old rat brain hypothalamic sections with anti-vimentin antibody showed that this type 3 intermediate filament was localised to cell soma in the ependymal wall and to processes that extended into the surrounding neuropil (Fig. 1B). Similarly, staining with an anti-GFAP antibody was observed in tanycytes cells of the sub-ependymal regions with a morphological appearance characteristic of astrocytes (Fig. 1C arrowheads). In primary cell cultures derived from the micro-dissected ependymal wall, all of the cells were found to express vimentin (Fig. 2A), whereas only a few cells were GFAP positive (Fig. 2B and C), which represented

either a small number of astrocytes having been carried over or a majority of tanycytes in culture having lost expression of GFAP. In comparison, cultures derived from the brain cortex extensively expressed both vimentin and GFAP markers (Fig. 2D,E and F). Interestingly, we observed that tanycyte cultures often had a greater cell density in contrast to cortical cell cultures, and this might be linked to the stem cell potential of tanycytes (Bolborea & Dale 2013). Primary cell cultures were assessed and compared to tissue explants for the expression of genes known to be mostly localised to tanycytes: *Dio2*, *Gpr50*, *Vimentin*, *Darpp-32* and the TSH receptor (Fig. 3). Transcripts for all of these genes were detected in both primary cell cultures and explants. To eliminate possible contamination by cells from the neighbouring pars tuberalis during the isolation procedure, PCR amplification with primers for TSH β was performed (Fig. 3), but no amplicons were detected for the hypothalamic explants or for the tanycytes cultures. However, as expected, TSH β was amplified from the rat pars distalis explants (Fig. 3).

TSHR cell signalling pathway activated in tanycyte cultures

The primary signal transduction pathway for TSH receptor is activation of adenylate cyclase via a G α_s G protein-coupled receptor (Allgeier et al. 1994, Laugwitz et al. 1996, Calebiro et al. 2010). When primary cell cultures were treated with TSH 1, 10 and 100 IU/l for 1 h, cAMP levels increased in a dose-dependent manner from an unstimulated value of 2.57 ± 0.07 – 2.92 ± 0.08 pmol/ml at 1 IU/l (ANOVA, post-hoc Tukey's, not significant), 3.59 ± 0.07 pmol/ml at 10 IU/l (ANOVA, post-hoc Tukey's, $P < 0.05$) and 4.12 ± 0.24 pmol/ml at 100 IU/l (ANOVA, post-hoc Tukey's, $P < 0.05$). This was a modest rise as compared to the maximal stimulation of cAMP levels that was reached by activating the adenylate cyclase with 10 mM forskolin (15.60 ± 0.33 pmol/ml; ANOVA, post-hoc Tukey's, $P < 0.05$; Fig. 4). Alternative G protein coupling was investigated using phosphorylation of ERK1/2 as a marker of receptor coupling to other G α proteins. Primary cell cultures were treated with forskolin (10 mM) or TSH (100 IU/l) for 5 min (Fig. 5A). In comparison to the control, forskolin had no effect, but TSH increased phosphorylation of ERK1/2 (p42/44, Fig. 5A). Phosphorylation of ERK1/2 also occurred in primary cell cultures that had been pretreated with cholera toxin for 16 h to eliminate coupling to G α_s protein (Fig. 5B). TSH stimulates *Dio2* in primary cell cultures. Primary cell cultures treated for 7 h with 100 IU/l TSH resulted in a significant 2.17-fold increase in *Dio2* mRNA expression as compared to the unstimulated control (t-test, $P < 0.001$; Fig. 6).

Discussion

The present study demonstrates that TSH receptors localised on ependymal cells of the hypothalamus transduce the signal of hormone binding via both activation of adenylate cyclase and phosphorylation of ERK1/2. Furthermore, activation of the TSH receptor leads to an increase in Dio2 mRNA expression. To investigate the signal transduction mechanism of the TSH receptor in the hypothalamic ependymal layer, we chose to utilise primary cell cultures of these cells from 10-day-old rat brains, as described by Prevot et al. (2003). First, as found in other species, TSH receptor mRNA expression was confirmed by in situ hybridisation in the hypothalamus of 10-day-old rats, confined to the cells adjacent to the third ventricle (Hanon et al. 2008, Nakao et al. 2008, Ono et al. 2008, Ross et al. 2011, Herwig et al. 2013). Using primary ependymal layer cell cultures prepared from 10-day-old rats, we investigated the signal transduction mechanism used by TSH receptors to transduce hormone binding in these cells. The cultures showed characteristics of tanycyte cells with the expression of mRNAs that are mostly restricted to tanycytes of the third ventricle, such as Gpr50, Darpp-32, Dio2 and TSHr (Ma et al. 1994, Barrett et al. 2006, Herwig et al. 2013), immunoreactivity for vimentin and no expression of TSH β mRNA (ruling out contamination of cells from the neighbouring pars tuberalis). Stimulation of the tanycyte primary cell cultures with TSH produced a dose-dependent increase in secreted cAMP levels. The functionality of the TSH receptor signalling was also evident, with a 2.17-fold increase in expression of Dio2 mRNA after treatment with TSH. The TSH receptor has been shown to couple with up to ten different G proteins representing members of all four families of G proteins (G α_i , G α_s , G $\alpha_q/11$ and G $\alpha_{12/13}$), thereby activating adenylate cyclase, phospholipase C and ERK1/2 (Laurent et al. 1987, Allgeier et al. 1994, Kursawe & Paschke 2007, Buch et al. 2008). However, the functional significance of this potential promiscuity of G protein coupling is not understood, and the outcome of TSH receptor activation is likely to be dependent on the cell type expressing the receptor and the repertoire of available G proteins. In our primary cell cultures, we show that TSH was able to stimulate adenylate cyclase, but it was also able to stimulate phosphorylation of ERK1/2. ERK1/2 is a common downstream effector of seven transmembrane domain receptors coupled to a range of G protein subtypes. ERK1/2 phosphorylation can occur via a pathway that involves a cascade from activation of protein kinase C leading to activation of Raf, which further activates mitogen-activated protein kinase to phosphorylating ERK1/2 via G proteins other than G α_s [reviewed Gutkind (2000) and Werry et al. (2005)]. However, adenylate cyclase activation by G α_s can also lead to ERK1/2 activation via both a protein kinase A-dependent and a protein kinase A-independent mechanism. In our primary cell

cultures, forskolin robustly activated adenylate cyclase, but this did not increase phosphorylation of ERK1/2, which suggests that the TSH receptor in our primary cell cultures was coupled to a $G_{\alpha s}$ protein for the generation of cAMP but also to another G protein to facilitate increased phosphorylation of ERK1/2. The relevance of a bifurcation of TSH receptor signalling in tanycytes is unknown. Furthermore, whether all tanycytes have a dual signalling pathway or whether there may be regionalisation in this capacity based on tanycyte subtype distribution in the third ventricle (Rodriguez et al. 2005) are intriguing questions because of the regionalisation of neuronal stem cell activity in response to the stimulus for proliferation (Bolborea & Dale 2013). TSH has been identified as the messenger from the pars tuberalis to act on ependymal tanycytes; it has been shown to increase CREB phosphorylation, Dio2 mRNA expression and local T3 production in seasonal mammals and birds and in non-seasonal mice (Hanon et al. 2008, Nakao et al. 2008, Ono et al. 2008, Unfried et al. 2009, Helfer et al. 2013). Although Sprague Dawley rats are not known to respond to photoperiods with altered physiology or behaviour, the potential for signalling of TSH secreted from the pars tuberalis is present in this rat strain with the presence of a functional TSH receptor located in the ventricular ependymal layer. However, the applicability of the present findings may be more generic rather than merely applicable to mammals that are normally associated with seasons, as most laboratory strains of rats have the potential to respond to photoperiods with physiological changes following olfactory bulbectomy (Nelson & Zucker 1981) or after manipulation of testosterone-negative feedback (Wallen et al. 1987). Moreover, the F344 rat strain does show physiological responses to photoperiods in terms of food intake and body weight (Ross et al. 2011) and increases in Dio2 expression in the ependymal layer following i.c.v. administration of TSH (Helfer et al. 2013). Furthermore, melatonin-proficient mice exposed to long-day photoperiods exhibit an appropriate response of TSH β up-regulation in the pars tuberalis and Dio2 expression in the ependymal layer. I.c.v. infusion of TSH into mice also increases CREB phosphorylation and Dio2 expression in the ependymal cell layer, which supports the view that TSH secreted from the pars tuberalis acts in a paracrine manner to regulate gene expression in tanycytes (Ono et al. 2008, Unfried et al. 2009). Consequently, TSH signalling regulated by photoperiod or other mechanisms may play a hitherto generic, but unknown, role in hypothalamic functions. Thus, even in laboratory animals that are generally considered to be non-photoperiodic, an evolutionarily ancient mechanism (Hanon et al. 2008) may be revealed whereby TSH determines the ability of tanycytes to regulate deiodinase activity and, hence, local thyroid hormone availability. This mechanism appears to be integral to the seasonal regulation of hypothalamic function (Bolborea & Dale 2013, Dardente et al. 2014), but it might also serve as a

convergence point for other inputs. For example, food restriction also increases Dio2 expression in rats (Diano et al. 1998) and in hamsters housed in short days (Herwig et al. 2009), but it remains to be determined whether this is also a TSH-driven process.

In summary, we demonstrated that in ependymal cell cultures, TSH leads to an increase in cAMP and Dio2 expression. Furthermore, we showed that TSH has the ability to activate an alternative signal transduction pathway through a cAMP-independent mechanism. This pathway merits further investigation, because it may be relevant in other unknown aspects of ependymal cell physiology that affect hypothalamic–neuroendocrine communication.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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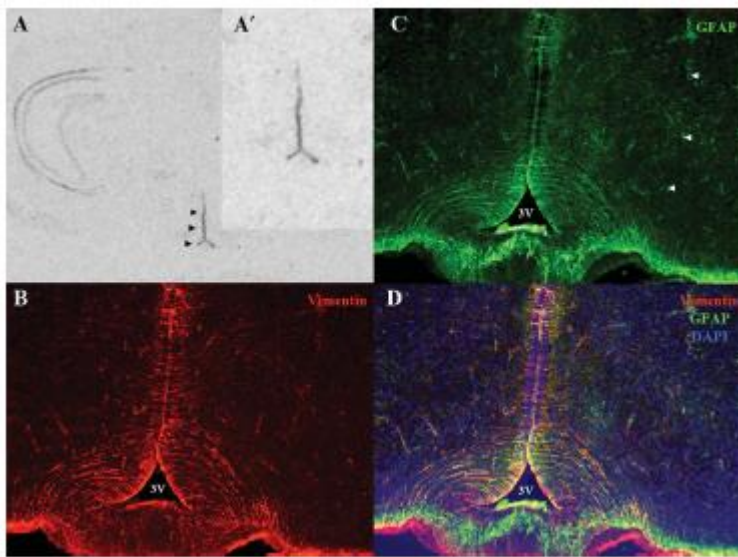


Figure 1

Localisation of TSH receptors, vimentin and GFAP at the interface of the ependymal layer and neuropil of 10-day-old rats. (A) In situ hybridisation for TSH receptors on a brain section of a 10-old-rat shows a high level of expression in the ventral region of the rat hypothalamus originating from cells that constitute mainly tanycytes (arrowheads). (A') Enlarged region over the area of the hypothalamus and third ventricle. (B and C) Immunohistochemistry for vimentin (B) and GFAP (C) on formaldehydefixed tissue at a ventral location of the hypothalamus in the region of the arcuate nucleus. (D) Merged image showing colocalisation of vimentin and GFAP; 3V, third ventricle, white arrowheads, GFAP (green) immunohistochemical staining indicative of astrocytes. (B, C and D) Micrographs taken at 40x magnification.

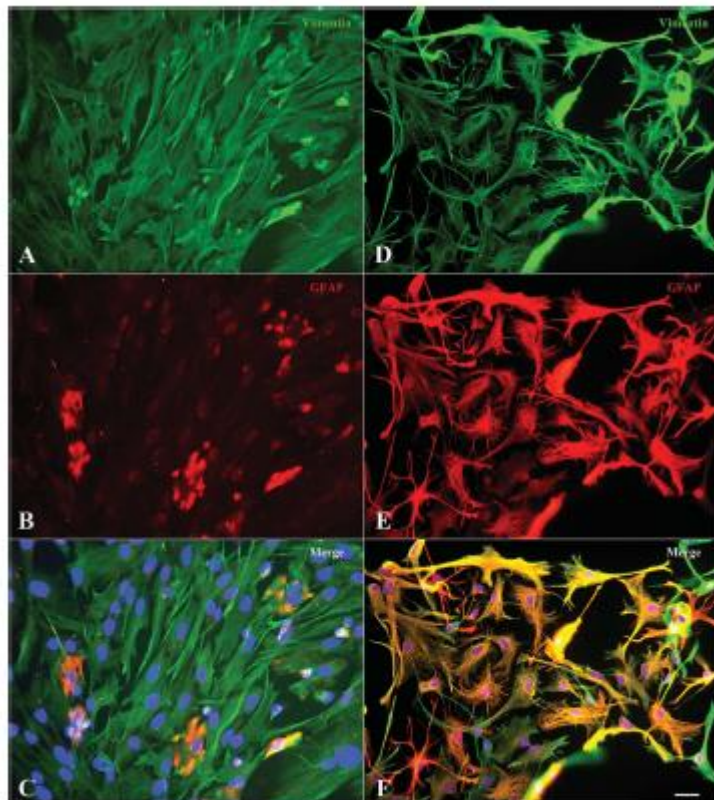


Figure 2

Immunocytochemical comparison of vimentin and GFAP on ependymal and cortical primary cell cultures. Vimentin immunocytochemistry on primary ependymal cell cultures (A) or primary cortical cultures (D). GFAP immunocytochemistry on primary ependymal cell cultures (B) or primary cortical cultures (E). Merged images of vimentin and GFAP and DAPI staining on primary ependymal cells (C) and cortical primary cell cultures (F). Scale: 25 μ m.

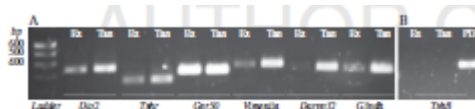


Figure 3

Expression of tanyocyte markers in primary ependymal cell cultures. (A) PCR amplification using cDNA reverse transcribed from RNA isolated from either hypothalamic tissue (E) or primary cell cultures (T) of type 2 deiodinase (Dio2), TSH receptor (TSHr), orphan G protein-coupled receptor

Gpr50 (Gpr50), vimentin and dopamine- and cAMP-regulated neuronal phosphoprotein (Darpp-32). Glyceraldehyde-3-phosphate dehydrogenase (G3pdh) was used as a housekeeping gene. (B) PCR amplification using cDNA reverse transcribed from RNA isolated from either hypothalamic tissue (Ex) or primary cell cultures (Tan) for the β subunit of TSH (TSH β) to test for contamination of tissue or cultures from cells of the pars tuberalis. RNA isolated from the pars distalis (PD) was used as a positive control.

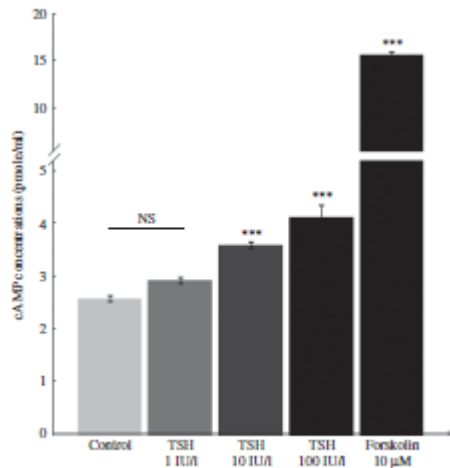


Figure 4

TSH increases cAMP production and regression from primary cell cultures. Primary cell cultures were serum deprived for 16 h before treatment with 1, 10 and 100 IU/l or 10 mM forskolin for 1 h. cAMP was determined in the cell culture media of treatments performed in triplicate, and shown is one representative of two independent experiments. ANOVA followed by post-hoc Tukey's. NS, not significant, *** $P < 0.05$.

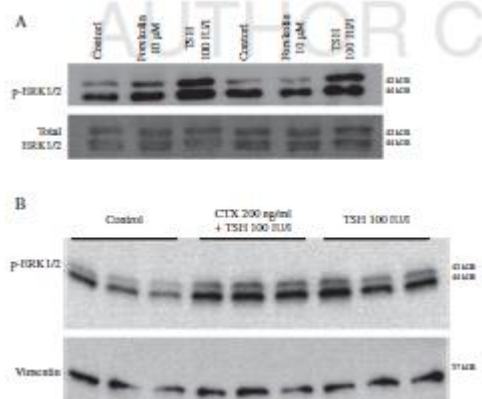


Figure 5

Stimulation of ERK1/2 phosphorylation by TSH in primary cell cultures. (A) 100 IU/l TSH increased ERK1/2 phosphorylation, whereas forskolin had little or no activity. (B) TSH 100 IU/l stimulated ERK1/2 phosphorylation in cholera toxin (CTX)-pretreated cells, which together with the absence of stimulatory activity by forskolin indicates that TSH stimulates ERK1/2 phosphorylation by a Gas-independent mechanism. Shown is one representative experiment of two independent experiments for each assay with treatments in duplicate or triplicate.

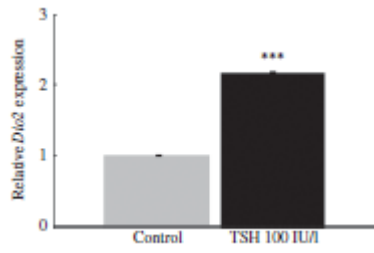


Figure 6

Quantitation of Dio2 mRNA expression by PCR in TSH-stimulated primary cell cultures. Treatment of primary cell cultures with 100 IU/l TSH stimulated a 2.17-fold increase in Dio2 mRNA expression. Treatments were performed in triplicate in two independent experiments. t-test: ***P<0.01.