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

Matei Bolborea[1; 2;*], Gisela Helfer[1], Francis J.P. Ebling[2], Perry Barrett[1]

[1] Rowett Institute of Nutrition and Health, University of Aberdeen, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB United Kingdom.

[2] School of Life Sciences, University of Nottingham Medical School, Nottingham, NG7 2UH United Kingdom

* Present address: School of Life Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL United Kingdom

Author for correspondence

Dr Perry Barrett

Rowett Institute of Nutrition and Health,

University of Aberdeen,

Greenburn Road,

Bucksburn,

Aberdeen, AB21 9SB

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**Abstract**

Tanycytes have multiple roles in hypothalamic functions including sensing of peripheral nutrients and metabolic hormones, regulation of neurosecretion and mediation of seasonal cycles of reproduction and metabolic physiology. This latter function reflects expression of thyroid hormone stimulating (TSH) receptors in tanycytes, which detect photoperiod-regulated changes in TSH secretion from the neighbouring *pars tuberalis*. Our 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 rat was observed by *in situ* hybridization. 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 tanycytes signals via $G\alpha_s$ to increase cAMP and 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 specialized ependymoglial cells called tanycytes. These cells have a distinct morphology. They interface with the cerebrospinal fluid (CSF) 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, 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 II deiodinase (Dio2) at very high levels, an enzyme that converts the weakly active form of thyroid hormone L-thyroxine (3,3',5,5'-tetraiodo-L-thyronine or T4) to the biologically active form, triiodothyronine (3,5,3'-triiodo-L-thyronine or T3). This enzyme is important in diverse physiological responses, which include fasting and non-thyroid illness caused by bacterial infections where in both cases tanycytic *Dio2* expression and T3 production is increased (Klosen *et al.* 2002; Sanchez *et al.* 2010). The importance of tanycytes as a source of T3 is also evident in seasonal mammals where 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, by an unknown mechanism (Coppola et al 2007), under inflammatory conditions, by a NFkappaB dependent signalling mechanism (de Vries et al 2014; Wittmann et al. 2014) and in long-day photoperiods, by thyroid stimulating hormone (TSH)** of *pars tuberalis* origin (Hanon *et al.* 2008; Nakao *et a.l.*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 of mainly tanycytes (Ross *et al.* 2011; Herwig *et al.* 2013). Intracerebral ventricular 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. Activation of adenylate cyclase is a signal transduction pathway commonly associated with TSH receptor activation, but TSH receptor are known to couple with a diverse range of G proteins activating several different pathways with potentially 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 as this may have further consequences for the function of tanycytes and their regulation of the surrounding hypothalamus. In this study, we used dissociated primary hypothalamic ependymal cell cultures of 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 hours / 12 hours) and constant temperature (20 ± 2°C) and humidity (55% ± 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 genders. Experimental procedures were approved by the Rowett Institute ethics committee, and animals were euthanized under Schedule 1 of the Animals (Scientific Procedures) Act 1986 UK.

### *Primary cell culture*

Brains were collected and micro-dissected in ice-cold Dulbecco's phosphate buffer saline solution (Sigma Aldrich) using a binocular magnifying microscope. The preparation of primary cell cultures was achieved

using the protocol as previously described by Prévot *et al.* and others (Ma *et al.* 1994; McCarthy & de Vellis 1980; Prevot *et al.* 2003; 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 twenty rat pups were pooled in ice-cold Dulbecco's modified Eagle medium (DMEM; Sigma Aldrich), then centrifuged for 1 minute at 1500g and the medium exchanged for fresh DMEM. Explant tissue was then scraped through a 20 µm mesh over a Petri dish containing DMEM (Sefar UK). The dissociated tissue was transferred to a centrifuge tube and spun for 5 minutes at 1500g. 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 25cm culture flask (Coring Costar or Sigma Aldrich UK) with an additional 5 mL of supplemented medium. Cultures were incubated in a humid atmosphere of 5% $CO_2$-95% air at 37°C. Once cells had adhered (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 95mM phosphate buffer (20mM $NaH_2PO_4$, 75mM $Na_2HPO_4$, pH7.4) and fixed for 24 hours 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 1X phosphate buffered saline (1X PBS -137mM NaCl, 2.7mM KCl, 10mM $Na_2HPO_4$, 2mM $KH_2PO_4$, pH7.4) and a final wash in 1X PBS + 0.3% Triton X-100 (Sigma-Aldrich) to facilitate cell permeability. This was followed by a 60 minute incubation in a blocking buffer, containing 1X PBS + 0.3% Triton X-100 + 5% bovine serum albumin (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 1X PBS and then incubated with the secondary antibodies, respectively raised against the appropriate species, and coupled to fluorescein (Vector labs) at 1:1000 or Northern Lights™637 (R&D Systems) at 1:2000 dilutions.**

*In situ hybridisation*

Brains were extracted identically as for cell cultures or immunohistochemistry but rapidly frozen on dry ice. *In situ* hybridization was performed on 14µm frozen sections of 10-day-old rats brain sections, as described previously (Shearer *et al.* 2012). We used a $^{35}$S-labelled anti-sense riboprobe for the TSH receptor as described recently (Herwig et al 2013). Following hybridization 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 hour recovery period, followed by an overnight serum deprivation (16 hours), 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 IU/L, 10 IU/L and 100 IU/L or forskolin at 10µM for 60 minutes before removing the media for the cAMP assay. A colorimetric ELISA assay (Arbor Assay) 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 hours. The cell cultures were then serum deprived for 16 hours (overnight). The following morning cells were rinsed three times with DMEM at 37°C. Cells were then incubated for 5

minutes at 37°C **with DMEM only**, bovine TSH in DMEM (1 IU/L, 10 IU/L and 100 IU/L) or forskolin (10 µM). Where cells were pre-treated 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 minutes treatment, 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 6 x 10 seconds bursts at 5 microns amplitude setting (MSE soniprep 150) to disrupt cells structure and shear DNA. This was followed by 5 minutes at 95-100°C to denature proteins. Twenty microliters of lysate was loaded onto a 7 x 8 cm 10% SDS polyacrylamide gel and electrophoresed at 150V for 2 to 3 hours to separate the protein components by molecular mass. Proteins were transferred to a PVDF membrane (Biorad Laboratories) using wet transfer apparatus (Biorad 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 1X Tris buffered saline (TBS; 20 mM Tris-HCl, 137 mM NaCl, pH 7.6). Antibodies were incubated overnight at 4°C in 1X TBS containing 5% bovine serum albumin with 0.1% Tween-20. Antibodies used were anti-phospho-ERK1/2 (New England Biolabs; 1:1000 dilution), an anti-ERK1/2 (New England Biolabs; 1:1000 dilution) or an anti-vimentin (Sigma-Aldrich; 1:5000 dilution). Following washes in 1X TBS with 0.1% Tween-20, an appropriate secondary antibody at a 1:2000 dilution, linked to horse radish peroxidase (New England Biolabs) was used the next day on the PVDF membrane for one hour. Proteins were visualized by chemiluminescence using Pierce Supersignal West Pico chemiluminescent substrate (Scientific Laboratory Supplies).

*Reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (q-PCR)*

Brain explants were dissected as described above for primary cell cultures. Total RNA was extracted from brain tissue or primary cell cultures using QIAshredder (Qiagen) and 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) and Bioanalyzer 2100 (Agilent Technologies). One microgram of total RNA was reverse transcribed using Superscript II (Invitrogen). Partial sequences of *Dio2*, *Tshr*, *Gpr50*, *Vimentin*, *Darpp32*, *G3pdh* and *Tshβ* were then amplified from the brain explants cDNA templates using the HotStart GoTaq kit (Promega) and following primers based on rat and mouse sequences in the Genbank database: *Dio2* (NM_031720) - forward: CTCTTCCTGGCGCTCTATGACTCG / reverse: TCCTCTTGGTTCCGGTGCTT, (494 bp); *Tshr* (NM_012888) - forward: TCCAGGGMCTATGCAATGAAC / reverse: CAGCCCGAGTGAGGTGGAGGA, (312 bp); *Gpr50* (NM_001191915) - forward: AAGCTCCGAAATTCTGGCAACA / reverse: ATGAGAGGGAGGACGAAGTGGATG, (434 bp); *Vimentin* (NM_031140) - forward: AGAACACCCGCACCAACGAGAAGG / reverse: ACGCAGGGCAGCRGTGAGGTC (521 bp); *Darpp32* (NM_138521) - forward: CTTCGGGAGCTKGGGTAYC / reverse: AGGGAAAGGCATTGGGGACTCTG (461 bp); *G3pdh* (AF106860) - forward: ACCACAGTCCATGCCATCAC / reverse: TCCACCACCCTGTTGCTGTA (451 bp); *Tshβ* (NM_013116) - forward: CCGAAGGGTATAAAATGAACAGAG / reverse: ACCAGATTGCATTGCCATTACAGT (505 bp). The cycling conditions used were 60°C for the annealing temperature for *Dio2*, *Vimentin*, *Darpp32*, *G3pdh*, *Tshβ* and 58°C for *Gpr50* and *Tshr* with 40 cycles. The resulting PCR amplification products were separated through 2.0% (w/v) agarose gels (Melford Laboratories) and stained with SYBR®Safe DNA gel stain (Invitrogen). To control for the product size, the samples were compared to a 100 bp size marker (Hyperladder IV, Bioline).

For q-PCR analysis, primary cultured cells were plated in 60 mm petri dishes at a density of 300 000 cells/dish. Forty eight hours later, cells were serum starved overnight then treated either with DMEM or DMEM containing bovine TSH (100 IU/L) for 7 hours at 37°C. Total RNA was extracted and reverse transcribed as described above. Five nanograms of cDNA was used as template in the qPCR reaction using QuantiFastTM SYBR® Green PCR kit (Qiagen) on a Thermal Cycler 7500 Fast Real Time PCR System (Applied Biosystems). We used a Qiagen validated primers for *β-Actin* (Quanti Tect ® Primmer Assay, Rn_Actb_1_SG) and *Dio2* (Rn_Dio2_2_SG) obtained from Qiagen. Each PCR procedure included a negative control reaction

without template and each sample was run in triplicate **with the experiment performed twice**. The reaction conditions were as follows: amplification 5 minutes at 95°C, 40 cycles of 10 sec 95°C, 30 sec 60°C and dissociation curve analysis 15 sec 95°C, 1 min 60°C and 15 sec 95°C. The *β-actin* housekeeping gene was used as reference for the relative quantification of *Dio2* calculated based on the $2^{-\Delta CT}$ method.

## Results

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

*In situ* hybridization 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 (Figure 1A and 1A').

### *Primary tanycyte cultures*

**Immunocytochemical staining of 10-day-old rat brain hypothalamic sections with anti-vimentin antibody showed this type III intermediate filament was localised to cell soma in the ependymal wall and to processes extending into the surrounding neuropil (Figure 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 (Figure 1C arrowheads). In primary cell cultures derived from the micro-dissected ependymal wall, all cells were found to express vimentin (Figure 2A), whereas only a few cells were GFAP positive (Figure 2B and 2C), either representing a small number of astrocytes carried over or a majority of tanycytes in culture losing expression of GFAP. In comparison, cultures derived from the brain cortex extensively expressed both markers: vimentin and GFAP (Figure 2D, 2E and 2F). Interestingly, we observed that tanycyte cultures often had a greater cell density in contrast to cortical cell cultures, which might be linked to their stem cell potential (Bolborea & Dale 2013).**

209 Primary cell cultures were assessed and compared to tissue explants for the expression of genes known to
210 be mostly localised to tanycytes: *Dio2*, *Gpr50*, *Vimentin*, *Darpp-32* and the *TSH-receptor* (Figure 3).
211 Transcripts for all these genes were detected in both primary cell cultures and explants. To eliminate
212 possible contamination by cells from the neighbouring *pars tuberalis* during the isolation procedure, PCR
213 amplification with primers for *Tshβ* was performed (Figure 3), but no amplicons were detected for the
214 hypothalamic explants or for the tanycytes cultures. However, as expected, *Tshβ* was amplified from the rat
215 *pars distalis* explants (Figure 3).

216

217 ***TSHR cell signalling pathway activated in tanycyte cultures***

218 The primary signal transduction pathway for TSH receptor is activation of adenylate cyclase via a $G\alpha_s$ G-
219 protein coupled receptor (Calebiro *et al.* 2010; Allgeier *et al.* 1994; Laugwitz *et al.* 1996). When primary cell
220 cultures were treated with TSH 1 IU/L, 10 IU/L and 100 IU/L for 1 hour, cAMP levels increased in a dose
221 dependant manner from an unstimulated value of 2.57 ± 0.07 pmol/mL to 2.92 ± 0.08 pmol/mL, at 1 IU/L
222 (ANOVA, post-hoc Tukey's; not significant), 3.59 ± 0.07 pmol/mL at 10 IU/L (ANOVA, post-hoc Tukey's; $p_{value}$
223 < 0.05), to 4.12 ± 0.24 pmol/mL at 100 IU/L (ANOVA, post-hoc Tukey's; $p_{value} < 0.05$). This was a modest rise
224 compared to the maximal stimulation of cAMP levels was reached by activating the adenylate cyclase with
225 10 µM forskolin (15.601 ± 0.33 pmol/mL; ANOVA, post-hoc Tukey's; $p_{value} < 0.05$; Figure 4). Alternative G
226 protein coupling was investigated using phosphorylation of ERK1/2 as a marker of receptor coupling to
227 others Gα proteins. Primary cell cultures were treated with forskolin (10 µM) or TSH (100 IU/L) for 5
228 minutes (Figure 5A). In comparison to the control, forskolin had no effect but TSH increased
229 phosphorylation of p42/44 (ERK1/2, Figure 5A). Phosphorylation of ERK1/2 also occurred in primary cell
230 cultures pre-treated with cholera toxin for 16 hours to eliminate coupling to $G\alpha_s$ protein (Figure 5B).

231

232 ***TSH stimulates Dio 2 in primary cell cultures***

Primary cell cultures for 7 hours with 100 IU/L TSH, resulted in a significant 2.17-fold increase in *Dio2* mRNA expression compared to the unstimulated control (t-test, $p_{value} < 0.001$; Figure 6).

## Discussion

This study demonstrates that TSH receptors localized 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 the increase in *Dio2* mRNA expression. To investigate the signal transduction mechanism of the TSH receptor (TSH-R) in the hypothalamic ependymal layer we chose to utilize primary cell cultures of these cells from 10-day-old rat brains as described by Prévot *et al.* (Prevot *et al.* 2003). Firstly, as found in other species *Tsh-r* mRNA expression was confirmed by *in situ* hybridization in the hypothalamus of 10-day-old rats and 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 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); no expression of *TSHβ* mRNA (ruling out contamination of cells from the neighbouring *pars tuberalis*); and immunoreactivity for vimentin.**

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 10 different G proteins representing members of all four families of G proteins ($G\alpha_i$, $G\alpha_s$, $G_{q/11}$ and $G_{12/13}$), activating adenylate cyclase, phophoslipase 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 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 involving a cascade from activation of protein kinase C leading to activation of Raf, further activating mitogen-activated protein kinase kinase to phosphorylating ERK1/2 via G proteins other than $G\alpha_s$(reviewed Gutkind 2000; Werry et al 2005). However, adenylate cyclase activation by $G\alpha_s$ can also lead to ERK1/2 activation via both a protein kinase A dependent and independent mechanism. In our primary cell cultures, forskolin robustly activated adenylate cyclase but this did not increase phosphorylation of ERK1/2, suggesting that the TSH receptor in our primary cell cultures was coupled to a $G\alpha_s$ protein for the generation of cAMP, but also another G protein to facilitate increased phosphorylation of ERK1/2.** The relevance of a bifurcation of TSH receptor signaling in tanycytes is unknown. Furthermore whether all tanycytes have a dual signaling pathway or whether there may be a regionalization in this capacity based on tanycyte subtype distribution in the third ventricle (Rodriguez *et al.* 2005) are intriguing questions given the regionalization 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 where 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 photoperiod 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 our findings may be more generic than merely to mammals which are normally associated with seasons as most laboratory strains of rats have potential to respond to photoperiod 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 photoperiod in terms of food intake and body weight (Ross *et al.* 2011) and an increase in *Dio2* expression in the ependymal layer following intracerebroventricular administration of TSH (Helfer *et al.* 2013). Furthermore, melatonin-proficient mice exposed to **long-day photoperiods exhibit an appropriate response of TSHβ upregulation in the *pars tuberalis* and *Dio2* expression in the ependymal layer. Intracerebroventricular infusion of TSH into mice also increases CREB phosphorylation and *Dio2* expression in the ependymal cell layer, supporting 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 have 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 (Hanon *et al.* 2008) mechanism can 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 seasonal regulation of hypothalamic function (Bolborea & Dale 2013; Dardente *et al.* 2014), but might also serve 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), though it remains to be determined whether this is also a TSH-driven process.

In summary, we have demonstrated that in ependymal cell cultures TSH leads to an increase in cAMP and *Dio 2* expression. Further we have shown that TSH has the ability to activate alternative signal transduction pathway through a cAMP independent mechanism. This pathway will need further investigations since it may be relevant in other unknown aspects of ependymal cell physiology affecting 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 legends**

**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* hybridization for *TSH receptors* on a brain section of a 10-old-rat show 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 3rd ventricle;** (B) Immunohistochemistry for vimentin and (C) GFAP on formaldehyde fixed tissue at a ventral location of the hypothalamus in the region of the arcuate nucleus. (D) **Merged image showing colocalization of vimentin and GFAP**. Indicated is the third ventricle (3V) and white arrowheads indicate GFAP (green) immunohistochemical staining indicative of astrocytes. **B-D: micrographs taken at 40X magnification**.

**Figure 2. Immunoctyochemical 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 (C) on primary ependymal cells and (F) cortical primary cell cultures. Scale: 25 µm.

**Figure 3. Expression of tanycyte 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*, *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 transcribe from RNA isolated from either hypothalamic tissue (Ex) or primary cell cultures (Tan) for the *beta subunit of TSH* (*Tshβ*) 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.

459

460 **Figure 4. TSH increases cAMP production and egression from primary cell cultures.** Primary cell cultures
461 were serum deprived for 16h prior to treatment with 1, 10 and 100 IU/L or 10 μM forskolin for 1 hour.
462 Cyclic AMP was determined in the cell culture media of treatments performed in triplicate and shown is
463 one representative **of two independent** experiments. ANOVA followed by post-hoc Tukey's; NS - not
464 significant. *** - $p_{value} < 0.05$.

465

466 **Figure 5. Stimulation of ERK1/2 phosphorylation by TSH in primary cell cultures.** (A) 100 IU/L TSH
467 increased ERK1/2 phosphorylation whereas forsoklin has little or no activity (B) TSH 100 IU/L stimulated
468 ERK1/2 phosphorylation in cholera toxin (CTX) pretreated cells which together with the absence of a
469 stimulatory activity by forskolin indicates TSH stimulates ERK1/2 phosphorylation by a $G\alpha_s$ independent
470 mechanism. Shown is one representative experiment **of two independent experiments for each assay,**
471 **with from treatments in duplicate or triplicate.**

472

473 **Figure 6. Quantitation of *Dio2* mRNA expression by PCR in TSH stimulated primary cell cultures.**
474 Treatment of primary cell cultures with 100 IU/L TSH stimulated a 2.17 fold increase in *Dio2* mRNA
475 expression. **Treatments were performed in triplicate in two independent experiments.** t-test *** - $p_{value} <$
476 0.01.

477