Glutamine and glutamic acid enhance thyroid-stimulating hormone β subunit mRNA expression in the rat pars tuberalis

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Abstract

Thyroid-stimulating hormone (TSH)-producing cells of the pars tuberalis (PT) display distinct characteristics that differ from those of the pars distalis (PD). The mRNA expression of *TSH β* and *α GSU* in PT has a circadian rhythm and is inhibited by melatonin via melatonin receptor type 1; however, the detailed regulatory mechanism for *TSH β* expression in the PT remains unclear. To identify the factors that affect PT, a microarray analysis was performed on laser-captured PT tissue to screen for genes coding for receptors that are abundantly expressed in the PT. In the PT, we found high expression of the *KA2*, which is an ionotropic glutamic acid receptor (iGluR). In addition, the amino acid transporter A2 (*ATA2*), also known as the glutamine transporter, and glutaminase (*GLS*), as well as *GLS2*, were highly expressed in

the PT compared to the PD. We examined the effects of glutamine and glutamic acid on *TSH β* expression and *α GSU* expression in PT slice cultures. L-Glutamine and L-glutamic acid significantly stimulated *TSH β* expression in PT slices after 2- and 4-h treatments, and the effect of L-glutamic acid was stronger than that of L-glutamine. In contrast, treatment with glutamine and glutamic acid did not affect *α GSU* expression in the PT or the expression of *TSH β* or *α GSU* in the PD. These results strongly suggest that glutamine is taken up by PT cells through *ATA2* and that glutamic acid locally converted from glutamine by GLs induces *TSH β* expression via the *KA2* in an autocrine and/or paracrine manner in the PT.

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Introduction

The pars tuberalis (PT), which comprises the rostral part of the anterior lobe of the pituitary gland that surrounds the median eminence as a thin cell layer, has characteristics different from those of the pars distalis (PD). The mammalian PT contains two cell types: hormone-producing cells and folliculo-stellate cells. In general, the hormone-producing cells in the PT are glycoprotein hormone cells, i.e. the thyroid-stimulating hormone (TSH) cells and gonado-tropic hormone cells (Rudolf *et al.* 1993). The hormone-producing cells in the PT vary according to species (Gross 1984). In rats, most of the hormone-producing cells in the PT are small and oval-shaped TSH-producing cells (Gross 1984) and they are characterized by spot-like TSH immunoreactivity on the Golgi apparatus (Sakai *et al.* 1992). TSH produced in the PT (PT-TSH) acts on the TSH receptors and regulates the expression of type 2 deiodinase (Dio2) in the ependymal cell layer of the mediobasal hypothalamus, which results in the regulation of GnRH release into the median eminence in seasonal animals (Yoshimura *et al.* 2003, Watanabe *et al.* 2004). This effect of PT-TSH has also been reported in mice, which are nonseasonal breeding animals (Ono *et al.* 2008).

The regulatory mechanisms of *TSH β* and *α GSU* mRNA expression and TSH release in the thyrotropes of PT (PT-TSH cells) are believed to be different from those in PD cells because PT-TSH cells do not express the pituitary-specific transcription factor (Pit-1), TSH-releasing hormone receptor (TRH-R), and thyroid hormone receptor beta 2 (TR- β 2; Bockmann *et al.* 1997). In addition, a high density of melatonin-binding sites has been observed in the PT of many species (Williams & Morgan 1988, Weaver *et al.* 1989), and melatonin receptor type 1 (MT1) is expressed within only *α GSU*- and *TSH β* -expressing cells in the rat PT (Klosen *et al.* 2002). Melatonin is exclusively secreted at night, and the duration of the melatonin signal corresponds to the duration of the dark period, thereby providing photoperiodic information to melatonin receptors (von Gall *et al.* 2002). This indicates that melatonin has physiological functions in the PT and that the PT might play an important role in the mediation of seasonal or circadian signals (Hazlerigg 2001). In fact, the duration of the photoperiod affects the structure of TSH cells and *TSH β* mRNA expression in the PT cells of the Djungarian hamster (Bergmann *et al.* 1989, Bockmann *et al.* 1996, Arai & Kameda 2004). We previously reported that expression of *TSH β* and *α GSU* mRNA in the PT exhibits diurnal variations and that chronic

administration of melatonin significantly suppresses *TSH β* and α GSU mRNA expression (Aizawa *et al.* 2007). Moreover, we found that melatonin treatment alters TSH immunoreactivity and the number of TSH cells in the rat PT (Sakamoto *et al.* 2000). Another group also reported that acute melatonin injection suppresses *TSH β* mRNA expression in the mouse PT (Unfried *et al.* 2009). Taken together, these findings suggest that *TSH β* and α GSU mRNA expression in the PT is negatively regulated by melatonin.

Although PT-TSH is believed to play a physiologically important role as a photoperiodic mediator, the mechanisms by which PT-TSH is regulated remain unclear. Therefore, we performed a whole-genome expression analysis of laser-captured rat PT tissue to screen for factors that might be involved in the regulation of PT-TSH. We found that the KA2 kainate receptor (*KA2*), which is an ionotropic glutamic acid receptor (iGluR; Herb *et al.* 1992, Hollmann & Heinemann 1994); amino acid transporter A2 (*ATA2*), which is also known as the glutamine transporter (Sugawara *et al.* 2000); glutaminase (*GLS*); and *GLS2* (de la Rosa *et al.* 2009) were highly expressed in the PT. Thus, we examined the effects of glutamic acid and glutamine on *TSH β* and α GSU mRNA expression and TSH secretion in the PT.

Materials and Methods

Animals

Male Wistar rats weighing 200–250 g were maintained under a 12 h light/12 h darkness cycle (light switched on at 0800 h) at room temperature ($23 \pm 2^\circ\text{C}$) with food and water *ad libitum*. The animals were killed at Zeitgeber time 4 (ZT4) for tissue culture experiments and at ZT6 for microarray experiments. All procedures were approved and performed in accordance with the Saitama University Committee on Animal Research.

Preparation of sections and laser microdissection

Brains were frozen in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA, USA) and stored at -80°C . Frozen, 20 μm thick serial frontal sections were cut and mounted on membrane slides (cat. no. 11505189; Leica Microsystems, Wetzlar, Germany). To prevent RNA degradation during sectioning, these slides were precoated with 40 μl RNAlater-ICE (Ambion, Austin, TX, USA) and stored at -20°C inside a cryostat. The sections were fixed in ice-cold acetone for 2 min, dehydrated with a graded ethanol series (75 and 50%) for 1 min each, and stained for 10 s with 0.1% Toluidine Blue (Sigma–Aldrich, St. Louis, MO, USA) dissolved in 50% ethanol RNase-free water. The sections were rinsed in RNase-free water, dehydrated in a graded ethanol series (50 and 75%) for 1 min each, immersed in 100% ethanol for 5 s, and dried using a cold-air dryer for 1 min in preparation for laser microdissection (LMD).

The PT, arcuate nucleus (Arc), and PD were dissected from stained sections using an LMD system (LMD 7000; Leica

Microsystems), and the cut sections were directly captured into 0.2 ml tube caps filled with RLT buffer (RNeasy Micro Kit; Qiagen) containing β -mercaptoethanol (Supplementary Figure 1A, B and C; see section on supplementary data given at the end of this article). Total RNA was isolated using the RNeasy Micro Kit according to the manufacturer's protocol, including on-column DNase treatment. RNase-free water (14 μl) was applied for elution. The integrity of the isolated total RNA was verified by high-resolution microcapillary electrophoresis using the RNA 6000 Pico LabChip and the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Agilent 2100 Bioanalyzer Software was used to analyze electropherograms and to quantify the 28S and 18S rRNA band intensities.

Gene microarray analysis

The total RNA (500 ng) from ten rats was pooled and used for the Oligo DNA microarray analysis using the 3D-Gene Rat Oligo chip 20k (Toray Industries, Tokyo, Japan). The total RNA was labeled with Cy5 using the Amino Allyl MessageAMP II aRNA Amplification Kit (Applied Biosystems, Foster City, CA, USA). Hybridization was performed according to the supplier's protocol (<http://www.3d-gene.com>). Hybridization signals were scanned using the ScanArray Express Scanner (PerkinElmer, San Jose, CA, USA) and processed using the GenePixPro version 5.0 (Molecular Devices, Sunnyvale, CA, USA). The raw data of each spot were normalized to the mean intensity of the background signal, which was determined according to the 95% confidence intervals of all blank spot signal intensities. Raw signal intensities that were more than two S.D. higher than the background signal intensity were considered valid. The detected signals for each gene were normalized by a global normalization method (the median of the detected signal intensity was adjusted to 25).

Brain slice preparation and slice culture

Rats were killed at ZT4 when the *TSH β* and α GSU mRNA expression in the rat PT reached its daily nadir (Aizawa *et al.* 2007). The brain was immediately removed and 600 μm thick coronal slices were cut using a vibratome (VT1200S; Leica Microsystems) in ice-cold Dulbecco's PBS (–). These slices were then trimmed to 5 $\times$ 5 mm that included the adjacent hypothalamic area of the PT. After preincubation in serum-free DMEM containing low glucose (5.56 mM) without L-glutamine (cat. no. 11054-020; Invitrogen) for 2 h, the medium was changed to DMEM containing low glucose with 1 mM L-glutamic acid (Sigma–Aldrich) or 1 mM L-glutamine (Sigma–Aldrich), and the incubation was continued for 2–8 h. The PD slices were cut to 1 mm thickness and incubated in the same manner. After incubation, slices were fixed with 4% paraformaldehyde (PFA) in 0.067 M PB, pH 7.4 containing 0.02% glutaraldehyde for 12 h at 4°C . The slices were immersed in PBS containing 30% sucrose for

20 h at 4 °C and frozen in Tissue-Tek Compound. Frozen, serial, 8 μ m thick frontal sections were cut and mounted on silane-coated slides for *in situ* hybridization (ISH). The incubated PD was immersed in Isogen (Nippon Gene, Tokyo, Japan) for quantitative PCR (qPCR).

ISH for mRNAs of TSH β , α GSU, MT1, and KA2

ISH was performed as described previously (Aizawa *et al.* 2007). Briefly, the sections were treated with 0.5 μ g/ml proteinase K for 30 min at 37 °C, fixed with 4% PFA, and washed for 3 min with PBS. After treatment with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, the sections were washed with PBS for 1 min. Digoxigenin (DIG)-labeled anti-sense and sense rat MT1 cRNA probes (GenBank accession no. NM_053676.1, position 1077–1485), rat KA2 cRNA probes (GenBank accession no. NM_031508.1, position 3123–3507), rat TSH β cRNA probes (GenBank accession no. M10902, position 146–388), and rat α GSU cRNA probes (GenBank accession no. J00757, position 26–607) were synthesized using a labeling kit (Roche Diagnostics) with SP6 or T7 RNA polymerase (Roche Diagnostics). The probes were diluted to 1 ng/ μ l with hybridization buffer (50% formamide, 3 $\times$ saline sodium citrate (SSC; 150 mM NaCl, 15 mM sodium citrate, pH 7.4), 0.12 M PB, pH 7.4, 1 $\times$ Denhardt's solution, 125 μ g/ml tRNA, 0.1 mg/ml sonicated salmon sperm DNA, and 10% dextran sulfate) and placed on the tissue sections. A sense cRNA probe was used as a negative control. The sections were covered with Parafilm (Pechiney Plastic Packing Inc., Chicago, IL, USA) and incubated for 16 h at 58 °C in a humidity chamber. The covers were removed by soaking the slides in 5 $\times$ SSC, and the sections were subsequently immersed in 2 $\times$ SSC containing 50% formamide for 30 min. The sections were then treated with tris–NaCl–EDTA (TNE; 10 mM Tris–HCl, pH 7.6, 500 mM NaCl, and 1 mM EDTA, pH 8.0) for 10 min and with RNase A (5 μ g/ml in TNE) for 30 min at 37 °C. Next, the sections were immersed in TNE for 10 min at 37 °C and washed with 2 $\times$ SSC for 20 min at 50 °C and 0.2 $\times$ SSC for 20 min, each twice at 50 °C. The sections were further incubated for 5 min in buffer-1 (100 mM Tris–HCl, 150 mM NaCl, and 0.01% Tween 20, pH 7.5), immersed in 1.5% blocking reagent (Roche Diagnostics) in buffer-1 for 1 h at 37 °C, and subsequently washed in buffer-1 for 5 min. After washing, the sections were incubated with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) diluted 1:1000 in buffer-1. The sections were washed in buffer-1 for 15 min twice and in buffer-2 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl₂) for 3 min. A chromagen solution (337 μ g/ml 4-nitroblue tetrazolium chloride and 175 μ g/ml 5-bromo-4-chloro-3-indolyl-phosphate in buffer-2) was added, and the sections were incubated until a visible signal was detected. The reaction was stopped by adding a reaction stop solution (10 mM Tris–HCl, pH 7.6 and 1 mM EDTA, pH 8.0). The sections were washed with PBS and covered with 90% glycerol in PBS.

Immunohistochemistry for TSH

The immunohistochemical detection of TSH using rabbit anti-rat TSH serum (HAC-RT29-01RBP86; a gift from the Laboratory of Biosignal Sciences, Institute for Molecular and Cellular Regulation, Gunma University) was performed using the avidin–biotin complex (ABC) method. The production and specificity of the antibody have been described elsewhere (Kawarai 1980, Wakabayashi & Tanaka 1988). The sections were treated with 0.5% sodium metaperiodate to block endogenous peroxidase for 15 min at room temperature and were incubated with TNBS (1% normal horse serum and 0.4% Triton X-100 in PBS) for 1 h. After washing with PBS, the sections were incubated overnight with anti-TSH serum diluted 1:40 000 in TNBS in a humidified chamber. The ABC method was used for immunohistochemistry (IHC) using a staining kit (Vectastain ABC kit, Vector, Burlingame, CA, USA). All incubations were performed in a humidified chamber at room temperature. The reactions were visualized with 0.02% 3/3'-diaminobenzidine tetrachloride in 0.006% H₂O₂ in 50 mM Tris–HCl, pH 7.6.

RNA extraction and qPCR

The total RNA from the LMD-captured PT, Arc, and PD was extracted using the LMD and RNeasy Micro Kit mentioned above. The total RNA from the cultured PD was extracted using an Isogen Kit according to the manufacturer's instructions. The cDNA was synthesized from 60 ng total RNA from the LMD-captured tissues using the High Capacity RNA-to-cDNA kit (Applied Biosystems). The cDNA of the cultured PD was synthesized from 1 μ g DNase-treated total RNA with random primers using ReverTra Ace (Toyobo, Osaka, Japan). The primers used in this study are summarized in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. The qPCR reactions were performed using a LightCycler (Roche Diagnostics) with SYBR Premix Ex Taq (TakaraBio, Shiga, Japan). The initial template denaturation was programmed for 30 s at 95 °C. PCR was performed with 40 cycles of 5 s at 95 °C and 15 s at 60 °C, and a final cooling step was performed for 30 s at 40 °C. Rat GAPDH mRNA was used as the invariant control. The expression of each mRNA is shown relative to GAPDH mRNA expression. All reactions were performed in duplicate, and each transcript was quantitatively measured by establishing a linear amplification curve from serial dilutions of each plasmid containing the amplicon sequence. The amplicon size and specificity were confirmed by a melting curve analysis and 2% agarose gel electrophoresis.

In vitro TSH secretion from PT and PD

Brain slices were prepared using the same method as described above. Coronal slices (600 μ m thick) were trimmed to 4 $\times$ 2 mm squares that included the adjacent median eminence with the PT. Three slices of the PT from one brain were

Table 1 Candidate high-expressing genes in pars tuberalis (PT)

Symbol	Gene ID	Ensemble ID	Signal intensity		
			PT	PD	Arc
<i>PTPRN</i>	Receptor-type tyrosine-protein phosphatase-like N precursor (R-PTP-N)	ENSRNOG00000019587	7074.3	5779.8	2879.4
<i>RARRES2</i>	Retinoic acid receptor responder (tazarotene induced) 2	ENSRNOG00000024705	5198.5	614.3	246.7
<i>SPNA2</i>	Spectrin alpha chain, brain (alpha-II spectrin; Fodrin alpha chain)	ENSRNOG00000015396	4752.7	5877.1	1701.0
<i>DCN</i>	Decorin precursor (bone proteoglycan II)	ENSRNOG00000004554	4584.5	59.4	900.8
<i>KDELRL1</i>	ER lumen protein retaining receptor 1 (KDEL receptor 1)	ENSRNOG000000021082	4317.5	2417.4	2080.7
<i>RT1-A1</i>	RT1 class Ia, locus A1	ENSRNOG000000038999	4211.0	600.1	1290.3
<i>FCGRT</i>	IgG receptor FcRn large subunit p51 precursor (FcRn)	ENSRNOG00000020583	3831.1	327.9	205.8
<i>ANXA11</i>	Annexin A11	ENSRNOG00000010984	3305.6	660.0	1468.2
<i>KRT2-8</i>	Keratin, type II cytoskeletal 8 (CK-8)	ENSRNOG00000009779	3081.7	31.5	1352.5
<i>LR8</i>	Transmembrane protein 176B (protein LR8)	ENSRNOG00000008465	2944.3	1508.1	2061.1
<i>MEA1</i>	Male-enhanced antigen	ENSRNOG00000017144	2929.1	6413.2	2378.1
<i>RT1-KE4</i>	RT1 class I, locus Ke4	ENSRNOG00000000465	2853.1	1557.8	1889.8
<i>MXRA8</i>	Matrix-remodeling-associated protein 8 precursor (limitrin)	ENSRNOG00000019244	2820.3	343.4	223.1
<i>LRP10</i>	Low-density lipoprotein receptor-related protein 10	ENSRNOG00000011592	2591.1	873.4	737.9
<i>STX4A</i>	Syntaxin-4	ENSRNOG00000019302	2454.4	547.2	755.0
<i>HES6</i>	Hairy and enhancer of split 6 (<i>Drosophila</i>)	ENSRNOG000000020194	2422.0	1996.1	1734.1
<i>LGALS3BP</i>	Lectin, galactoside-binding, soluble, 3 binding protein	ENSRNOG00000003217	2237.3	469.6	206.1
<i>RT1-A2</i>	RT1 class Ia, locus A2	ENSRNOG000000030712	2134.9	268.6	1866.8
<i>GRINL1A</i>	Glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A	ENSRNOG00000016300	2013.7	3425.1	1636.9
<i>KA2</i>	Glutamate receptor KA-2 (glutamate receptor, ionotropic kainate 5)	ENSRNOG000000020310	1979.9	3166.7	2474.1
<i>PDGFRB</i>	Beta-type platelet-derived growth factor receptor precursor	ENSRNOG00000018461	1973.8	173.6	87.6
<i>MTVR2</i>	Mammary tumor virus receptor 2	ENSRNOG000000024239	1938.5	1687.7	1094.4
<i>Q64548-2</i>	RTN1_RAT isoform RTN1-S of Q64548 – <i>Rattus norvegicus</i>	ENSRNOG000000004794	1920.2	16123.5	2641.3
<i>RAMP2</i>	Receptor activity-modifying protein 2 precursor	ENSRNOG00000020415	1891.5	751.3	198.7
<i>NP_001099762.1</i>	Eukaryotic translation initiation factor 3, subunit 5 (epsilon)	ENSRNOG00000015221	1756.9	1739.0	1585.7
<i>CKRY</i>	Complement regulatory protein Crry precursor (antigen 512)	ENSRNOG00000008193	1740.4	454.7	812.2
<i>BCAP31</i>	B-cell receptor-associated protein 31	ENSRNOG00000019053	1722.6	1941.9	1200.4
<i>ADORA2B</i>	Adenosine receptor A2b	ENSRNOG00000002922	1663.3	91.2	28.7
<i>THRA_V2</i>	Thyroid hormone receptor alpha (C-erbA-alpha)	ENSRNOG00000009066	1649.1	3112.5	1213.7
<i>LOC688289</i>	EGF-containing fibulin-like extracellular matrix protein 2	ENSRNOG000000020587	1622.6	259.9	168.6
<i>LTBR</i>	Lymphotoxin B receptor	ENSRNOG00000019264	1605.4	494.4	535.3
<i>BOC</i>	Biregional cell adhesion molecule-related/down-regulated by oncogenes (Cdon) binding protein	ENSRNOG00000002041	1559.7	102.9	140.0
<i>OTUD5</i>	OTU domain-containing protein 5	ENSRNOG00000008764	1556.9	1349.5	1007.5
<i>ATP6AP2</i>	Renin receptor precursor	ENSRNOG00000003858	1555.8	5122.2	3255.8
<i>RAMP1</i>	Receptor activity-modifying protein 1 precursor	ENSRNOG000000019926	1552.5	1624.6	934.5
<i>LSR</i>	Lipolysis-stimulated lipoprotein receptor precursor	ENSRNOG000000021053	1488.2	155.9	1131.6
<i>NP_001099400.1</i>	Ataxin 2	ENSRNOG000000001256	1467.7	1780.4	546.3
<i>LGALS3</i>	Galectin-3 (carbohydrate-binding protein 35)	ENSRNOG000000010645	1450.8	313.9	155.5
<i>BRD2</i>	Bromodomain-containing protein 2 (protein RING3)	ENSRNOG000000000461	1442.7	941.0	714.0
<i>VCAM1</i>	Vascular cell adhesion protein 1 precursor (V-CAM 1)	ENSRNOG00000014333	1436.3	925.4	147.2
<i>PHB2</i>	Prohibitin-2 (B-cell receptor-associated protein BAP37)	ENSRNOG00000012999	1429.6	1269.6	1035.9
<i>SRA1</i>	Steroid receptor RNA activator 1 (steroid receptor RNA activator protein)	ENSRNOG00000018089	1394.5	1045.1	1146.1
<i>DKK3</i>	Dickkopf homolog 3	ENSRNOG00000016343	1286.9	1328.1	16.5
<i>RAD23B</i>	U.v. excision repair protein RAD23 homolog B	ENSRNOG000000016137	1276.2	1511.6	1033.3
<i>ZFR_RAT</i>	Zinc finger RNA-binding protein	ENSRNOG00000011627	1275.7	4442.0	2889.4
<i>LMBRD1</i>	LMBR1 domain-containing protein 1 (liver regeneration p53-related protein)	ENSRNOG000000012178	1264.7	1986.4	786.9
<i>CXCL16</i>	Similar to chemokine (C-X-C motif) ligand 16 (Cxcl16)	ENSRNOG000000026647	1262.5	545.9	274.6
<i>UBXN4</i>	UBX domain containing 2	ENSRNOG00000003625	1222.9	1584.4	1167.0
<i>AGPAT1</i>	1-Acylglycerol-3-phosphate O-acyltransferase 1	ENSRNOG000000000437	1221.1	1499.5	567.9
<i>CUTA</i>	Protein CutA precursor (brain acetylcholinesterase putative membrane anchor)	ENSRNOG000000000481	1206.7	1030.5	476.8
<i>GIT2</i>	G protein-coupled receptor kinase-interactor 2	ENSRNOG000000001190	1193.1	458.0	458.8
<i>THRAP4</i>	Mediator of RNA polymerase II transcription subunit 24	ENSRNOG000000008711	1149.9	848.8	619.9
<i>SS18</i>	Ss18 protein	ENSRNOG00000016800	1129.0	238.4	423.9
<i>ITPR1</i>	Inositol 1,4,5-trisphosphate receptor type 1 (type 1 InsP3 receptor)	ENSRNOG000000007104	1108.2	628.8	688.6
<i>SYVN1</i>	Syvn1 protein	ENSRNOG000000020950	1101.8	552.3	497.9
<i>RAB2L</i>	RAB2, member RAS oncogene family-like	ENSRNOG000000000474	1098.7	275.4	258.0
<i>HYAL2</i>	Hyaluronidase-2 precursor (Hyal-2)	ENSRNOG000000031420	1098.0	725.2	408.8
<i>RBM26</i>	RNA-binding protein 26 (RNA-binding motif protein 26)	ENSRNOG000000009836	1058.8	645.9	915.1
<i>MT1</i>	Melatonin receptor type 1 (MT1)	ENSRNOG000000028744	1032.5	4.6	7.2

Figure 1 *MT1* and *KA2* mRNA expression in the PT, PD, and Arc of male Wistar rats. (A) qPCR for *MT1* in the PT, PD, and Arc using LMD samples. High *MT1* expression was detected only in the PT. All values are means $\pm$ s.e.m. ($n=3$). (B) *KA2* mRNA expression in the PT, PD, and Arc. The *KA2* mRNA level in the PT was similar to that in the PD. Although there was no statistically significant difference in its mRNA levels between the PT and Arc, the *KA2* mRNA level tended to be lower in the PT ($P=0.057$). All values are means $\pm$ s.e.m. ($n=3$). (C) Microphotograph of *MT1* mRNA-expressing cells detected by ISH. *MT1* mRNA-expressing cells were restricted to the PT (arrows). (D) Microphotograph of *KA2* mRNA-expressing cells detected by ISH. *KA2* mRNA-expressing cells were observed not only in the PT (arrows) but also in the hypothalamus, including the Arc (arrowheads). Scale bar: 200 μ m. PT, pars tuberalis; Arc, arcuate nucleus; and 3V, third ventricle. Full color version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0388>.

incubated in 50 μ l culture medium (serum-free DMEM containing low glucose without L-glutamine, cat. No. 11054-020; Invitrogen) using a noncoated 96 well plate. The PD slices were also cut into a thickness of 1 mm and incubated in 200 μ l culture medium using a noncoated 48 well plate. After preincubation for 2 h, the brain slices were incubated in culture medium with 1 mM L-glutamine (Sigma-Aldrich) or 1 mM L-glutamic acid (Sigma-Aldrich) for 4 h. As a control, L-glutamine-free and L-glutamic acid-free culture media were both used. The media were subsequently centrifuged at 1000 g for 5 min, and the supernatants were collected and stored at -80°C until analysis. The TSH concentrations in the media were measured using an Lbis Rat TSH ELISA Kit (code no. AKRTS-010R; Shibayagi, Gunma, Japan) according to the manufacturer's instructions.

Morphological analysis

Sections stained for ISH and IHC were observed under a light microscope (BX60; Olympus, Tokyo, Japan) and photographed with a digital camera (DP70; Olympus). The digital images were transformed into gray scale by Adobe Photoshop (Adobe Systems), and a statistical analysis was carried out for the mean density per pixel measured using Scion Image (Scion, Frederick, MD, USA).

Statistical analysis

The values are given as the means $\pm$ s.e.m. Differences among groups were evaluated by a one-way ANOVA with Tukey's *post-hoc* tests using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Differences with $P<0.05$ were considered significant.

Figure 2 QPCR analysis of glutamic acid signaling components in the PT, PD, and Arc. (A) Glutamine transporter *ATA2* mRNA expression in the PT, PD, and Arc. The *ATA2* mRNA level was significantly higher in the PT than in the PD and Arc. (B) *GLS* mRNA expression in the PT, PD, and Arc. *GLS* mRNA expression was higher in the PT than in the PD and Arc. (C) *GLS2* mRNA expression in the PT, PD, and Arc. *GLS2* mRNA expression in the PT was significantly higher than that in the PD but comparable to that in the Arc. Values are the means $\pm$ s.e.m. ($n=3$). * $P<0.05$ and *** $P<0.001$.

Results

iGluR KA2 expression in the PT

To identify candidate genes in this microarray study using the dataset of molecular functions for Gene Ontology (<http://www.geneontology.org/>), we screened for genes that had 'receptor activity'. According to our results, 59 genes exhibiting higher expression levels than *MT1* (microarray signal intensity: 1032.5) were identified as candidate receptor genes with high expression in PT (Table 1). The adenosine receptor *A2B* gene, which is expressed in the PT (Rivkees & Reppert 1992, Stehle *et al.* 1992), was found among these genes (microarray signal intensity: 1663.3; Table 1). The expression of the *MT1* and adenosine receptor *A2B* genes was restricted in the PT, and their expression in the PD and Arc was notably low, i.e. not detected using this chip. Using this dataset, we first found that the expression of the *KA2* was high in the PT. The expression of *KA2* (microarray signal intensity: 1979.9) was higher than that of *MT1* and the adenosine receptor *A2B* (Table 1). In contrast, a high signal intensity for the *KA2* was detected not only in PT but also in PD (microarray signal intensity: 2474.1) and Arc (microarray signal intensity: 3166.7; Table 1).

For this study, qPCR and ISH were performed for *MT1* and *KA2* to validate the dataset obtained by the microarray analysis (Fig. 1). *MT1* expression was only detected in the PT (Fig. 1A), and the expression of the *KA2* in the PT and PD

was similar and tended to be lower than that in the Arc (Fig. 1B). *MT1* mRNA-expressing cells were observed only in the PT, whereas *KA2* mRNA-expressing cells were observed in the PT and the entire hypothalamus, including the Arc (Fig. 1C and D). A sense RNA probe to either the *MT1* or *KA2* generated no specific signal (data not shown). In addition, the microarray data showed that the expression of the other iGluRs and metabotropic GluRs (mGluRs) was low in the PT and that the NMDA receptor *NR2C*, *KA1* receptor, AMPA receptor *GLUR2*, and *MGLUR1* were expressed highly in the Arc. *GLUR2* and *KA2* expression was also detected in the PD (data not shown).

Glutamic acid signaling molecules in the PT

Glutamic acid is locally synthesized from glutamine and acts on GluRs as an autocrine and/or paracrine extracellular signal mediator in the central nervous system (CNS) and peripheral tissues. To determine whether glutamic acid synthesis occurs in the PT, we analyzed the expression of the molecules involved in glutamic acid synthesis, i.e. the glutamine transporter and Gls. In the microarray dataset, *ATA2*, which is a glutamine transporter, was detected in the PT (data not shown). The expression of *ATA2* (microarray signal intensity: 2460.6) was much higher in the PT than in the PD (microarray signal intensity: 539.5) and Arc (microarray signal intensity: 625.2). Moreover, qPCR revealed that the

Figure 3 Effects of glutamine and glutamic acid on *TSH β* mRNA expression in the PT, as determined by ISH. (A) Microphotographs of ISH for *TSH β* mRNA. The staining densities of *TSH β* mRNA were altered by treatment with glutamine (middle column) or glutamic acid (right column) compared to the control (left column) at each time point. Scale bar = 200 μ m. 3V, third ventricle. (B) Morphometric analysis showing that treatment with glutamine or glutamic acid significantly increased *TSH β* mRNA expression compared to the control at 2 and 4 h. The effect of glutamic acid on *TSH β* mRNA expression was significantly stronger than that of glutamine at 2 and 4 h. Values are the means $\pm$ S.E.M. ($n=4$). ** $P<0.01$ and *** $P<0.001$. Full color version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0388>.

expression of *ATA2* was significantly higher in the PT than in the PD and Arc ($P < 0.001$; Fig. 2A). In addition, the expression of *GLS* and *GLS2* was also detected in the PT, and the expression of *GLS* was higher in the PT than in the PD and Arc ($P < 0.05$; Fig. 2B). *GLS2* expression was significantly higher in the PT than in the PD but was comparable to that in Arc ($P < 0.05$, PT vs PD; Fig. 2C).

Effects of glutamine and glutamic acid on PT-TSH

To determine the effect of glutamine or glutamic acid on the expression of *TSH β* and α GSU mRNA in the PT, slice culture experiments were performed (Figs 3 and 4). In the control, the signal density of ISH for *TSH β* mRNA was low at 0 h and increased slightly at 8 h (Fig. 3A, left column). In contrast, treatment with glutamine increased the staining density in a time-dependent manner, and strong staining was detected at 4 and 8 h (Fig. 3A, middle column). Moreover, glutamic acid treatment strongly intensified the staining density at 2 and 4 h; however, the staining density decreased at 8 h (Fig. 3A, right column). A statistical analysis of the images showed that glutamine and glutamic acid significantly increased the *TSH β* mRNA expression compared to the control at 2 and 4 h (Fig. 3B). Moreover, the *TSH β* mRNA expression at 2 and 4 h after glutamic acid treatment was significantly higher than those after glutamine treatment ($P < 0.01$; Fig. 3B). In contrast, neither glutamine nor

glutamic acid stimulated the expression of α GSU in the PT (Fig. 4A and B). The TSH immunoreactivity in the PT was not affected by the addition of glutamine or glutamic acid to the medium (Fig. 5), and spot-like staining of the Golgi apparatus was observed in all examined conditions (Fig. 5A). However, after 8 h of incubation with glutamic acid, strong spot-like staining of the Golgi apparatus was observed, which was consistent with the control, but weak cytoplasmic staining was also observed (Fig. 5A, right bottom); this staining density was significantly lower than that of the control ($P < 0.01$) and glutamine-treated samples ($P < 0.05$; Fig. 5B). Additionally, to analyze the TSH secretion from the PT, we performed an *in vitro* slice culture of the PT. Treatment with 1 mM glutamic acid significantly increased the TSH concentration in the medium compared to the control ($P < 0.05$; Fig. 5C). Although the difference was not statistically significant, glutamine treatment tended to increase the TSH concentration (Fig. 5C). Additionally, after treatment with glutamic acid or glutamine for 4 h, we treated the slices with 50 mM KCl to release the stored TSH in the cells. KCl treatment for 2 h did not induce TSH secretion (data not shown).

Effects of glutamine and glutamic acid on PD-TSH

Finally, we examined the effect of treatment with glutamic acid or glutamine on *TSH β* and α GSU expression in the

Figure 4 Effects of glutamine and glutamic acid on α GSU mRNA expression in the PT, as determined by ISH. (A) Microphotographs of ISH for α GSU mRNA. The staining density of α GSU mRNA was not changed by treatment with glutamine (middle column) or glutamic acid (right column) compared to the control (left column) at each time point. Scale bar = 200 μ m. 3V, third ventricle. (B) Morphometric analysis showing that treatment with glutamic acid or glutamine did not affect the staining density of α GSU in PT. Values are the means $\pm$ S.E.M. (n=4). Full color version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0388>.

Figure 5 Effects of glutamine and glutamic acid on TSH immunoreactivity and TSH secretion in the PT. (A) Microphotographs of TSH immunoreactivity determined by IHC. Strong spot-like staining of the Golgi apparatus was observed in all examined conditions. Strong spot-like staining of the Golgi apparatus was observed, consistent with that in the control, but weak cytoplasmic staining was observed 8 h after glutamic acid treatment. Scale bar = 50 μ m. 3V, third ventricle. (B) Morphometric analysis showed that the staining density was not altered at 0, 2, or 4 h after glutamine or glutamic acid treatment. After incubation with glutamic acid for 8 h, the TSH immunoreactivity in PT was significantly lower than that in the control or after glutamine treatment. * $P < 0.05$ and ** $P < 0.01$. Values are the means $\pm$ s.e.m. ($n = 4$). (C) ELISA measuring the concentration of TSH secreted into the medium from brain slices that included the PT. Treatment with 1 mM glutamic acid for 4 h significantly increased the TSH secretion compared to the control. * $P < 0.05$. Values are the means $\pm$ s.e.m. ($n = 3$). Full color version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0388>.

cultured PD by qPCR. No significant difference was observed in *TSH β* or *α GSU* expression in the PD (Fig. 6A and B). In addition, neither glutamine nor glutamic acid altered TSH secretion into the medium (Fig. 6C). However, 50 mM KCl stimulation significantly increased the TSH concentration (data not shown).

Discussion

TSH β and *α GSU* expression and TSH secretion have been thoroughly studied in the PD and are regulated by the TRH–TSH–thyroid hormone feedback loop (Shupnik & Ridgway 1985, Carr *et al.* 1989, Zoeller *et al.* 2007). In addition,

Figure 6 Effect of glutamine and glutamic acid on PD-TSH mRNA expression and PD-TSH secretion. (A) *TSH β* mRNA expression and (B) α GSU mRNA expression determined by qPCR were not altered by either treatment at either time point. The values are the means $\pm$ S.E.M. ($n=3$). (C) The TSH secretion in the PD was determined by ELISA. The TSH secretion was also not affected by each treatment. The values are the means $\pm$ S.E.M. ($n=3$).

vasopressin, somatostatin, and dopamine are involved in the regulation of *TSH β* and α GSU expression and TSH secretion in the PD through specific receptors (Krass *et al.* 1968, Samuels *et al.* 1992, Lam & Wong 1999). However, the regulatory mechanisms for TSH expression and secretion in the PT are believed to be different from those in the PD because TRH-R and TR- β 2 are not produced in PT-TSH cells (Bockmann *et al.* 1997), and the mRNAs of the receptors for vasopressin, somatostatin, and dopamine were not detected in the PT in our microarray analysis or by qPCR (data not shown). In fact, *TSH β* and α GSU mRNA expression exhibits diurnal variations, and the chronic administration of melatonin significantly suppresses *TSH β* and α GSU mRNA expression in the PT (Aizawa *et al.* 2007). The 5'-upstream region of the *TSH β* gene, including an E-box-like element and cAMP response-like element, can be upregulated by the clock proteins BMAL1 and CLOCK, which are expressed in the PT (Unfried *et al.* 2009). In addition, Eya3, which is a transcription factor expressed in the PT, and Six1 synergistically induce *TSH β* expression via the six consensus sequence, and the activation of the *TSH β* promoter is further enhanced by Tef and Hlf via the D-box (Masumoto *et al.* 2010). Thus, it has been suggested that these transcriptional factors, which are rhythmically expressed in the PT, regulate PT-*TSH β* mRNA expression.

In this study, we found that glutamic acid stimulates *TSH β* mRNA expression in the PT. Glutamic acid plays an important role not only as an excitatory amino acid neurotransmitter in the CNS but also as an extracellular signal mediator in peripheral tissues, including the PD (Hinoi *et al.* 2004). Specific, high-affinity binding sites for [3 H] glutamic acid are located in the pituitary gland and brain of the rat (Yoneda & Ogita 1986, Meeker *et al.* 1994). Molecular biological and immunohistochemical analyses have demonstrated the presence of non-NMDA (Kiyama *et al.* 1993, Mahesh *et al.* 1999) and NMDA iGluRs in the pituitary gland (Petrallia *et al.* 1994, Bhat *et al.* 1995). In addition, Hinoi *et al.* demonstrated that [3 H] kainate (KA), which is an agonist of KA receptors, binds to the rat pituitary gland. Hinoi *et al.* also showed that an i.p. injection of KA increases the DNA-binding activity of the nuclear transcription factor activator protein-1 (AP1) in the rat pituitary gland and hippocampus (Hinoi & Yoneda 2001). In isolated rat pituitary melanotrope, glutamic acid induces a marked increase in the cytosolic free Ca^{2+} concentration by a nonsynaptic mechanism (Giovannucci & Stuenkel 1995). Collectively, these findings indicate that particular subtypes of GluRs are functionally expressed in the pituitary gland. Several studies have demonstrated the direct regulation of hormone secretion by glutamic acid in primary anterior pituitary cells, e.g. the secretion of prolactin is induced by 1 mM glutamic acid (Pampillo *et al.* 2002), and its effect is blocked by MK-801, which is an antagonist of NMDA iGluRs (Login 1990). NMDA, which is an agonist of NMDA receptors, and KA both induce the secretion of GH (Niimi *et al.* 1994), and KA

stimulates LH and FSH release from the anterior pituitary gland (Zanisi *et al.* 1994).

Although many studies have demonstrated the physiological effect of glutamic acid on the PD, the effects of glutamic acid on the PT, including the expression of GluRs and the synthesis pathway of glutamic acid, have not yet been established. In this study, we showed that the mRNA expression of the iGluR *K42* in the PT was higher than that of *MT1* (Williams & Morgan 1988, Weaver *et al.* 1989) and the adenosine receptor *A2B* (Rivkees & Reppert 1992, Stehle *et al.* 1992). Our microarray analysis showed that among GluRs, only *K42* was expressed in the PT. Moreover, our slice culture experiment indicated that glutamic acid significantly enhanced *TSH β* mRNA expression at 2 and 4 h, whereas glutamic acid did not alter the expression of α *GSU* mRNA in the PT or the expression of *TSH β* or α *GSU* in the PD. Taken together, these results suggest that the regulatory mechanisms of *TSH β* mRNA expression are different in the PT and PD, and that glutamic acid is necessary for the maintenance of *TSH β* expression in the PT. Although glutamic acid significantly induced *TSH β* mRNA expression, we did not observe any corresponding staining differences in TSH immunoreactivity. However, the TSH secretion from the PT was significantly increased by the glutamic acid treatment, and this finding was consistent with the observed *TSH β* mRNA expression level. Most of the hormone-producing cells in the rat PT are small and oval-shaped TSH-producing cells (Gross 1984) and are characterized by spot-like TSH immunoreactivity on the Golgi apparatus, with few secretory granules being found in the cytoplasm under electron microscopic observation (Sakai *et al.* 1992), which suggests that TSH may be secreted constitutively from PT-TSH cells. In this study, spot-like TSH-immunoreactive staining of the Golgi apparatus was observed in all examined conditions (Fig. 5A). Furthermore, KCl treatment of the PT slice cultures did not stimulate the secretion of TSH into the medium (data not shown). Taken together, these results suggest that glutamic acid induces *TSH β* mRNA transcription and increases TSH production, and that TSH is immediately and constitutively secreted without accumulating in the cells.

Most glutamic acid obtained from food is consumed by the gut as the metabolic fuel, and little glutamic acid enters the blood stream (Reeds *et al.* 1996, 2000). The circulating glutamic acid concentration is low (0.07–0.1 mM in the plasma; Hawkins *et al.* 1995, Wang *et al.* 2007), which indicates that circulating glutamic acid does not physiologically interact with GluRs. In general, glutamic acid is locally converted by Gls from glutamine, which is absorbed into the cell via glutamine transporters (de la Rosa *et al.* 2009), acts as an autocrine and/or paracrine neurotransmitter in the CNS, and functions as an extracellular signal mediator in the peripheral tissues (Hinoi *et al.* 2004). The plasma concentration of glutamine generally ranges from 0.5 to 1.0 mM and may change in certain physiological states (Wang *et al.* 2007). Therefore, the 1 mM concentrations of glutamine

used in this study are reasonable and reflect the physiological conditions *in vivo*. We demonstrated that the *ATA2* expression in the PT was much higher than that in the PD and Arc. In addition, *GLS* and *GLS2* were expressed in the PT, and glutamine induced a time-dependent increase in *TSH β* mRNA expression in the PT. These results indicate that the glutamic acid synthesis pathway is active in the PT and that glutamic acid that is locally converted from glutamine in the PT stimulates *TSH β* expression in an autocrine and/or paracrine manner.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-11-0388>.

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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