Hypothalamo-hypophysial system in rats with autotransplantation of the adrenal cortex

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Abstract. Patients with bilateral pheochromocytoma often require an adrenalectomy. Autotransplantation of the adrenal cortex is an alternative therapy that could potentially be performed instead of receiving glucocorticoid replacement following adrenalectomy. Adrenal cortex autotransplantation aims to avoid the side effects of long-term steroid treatment and adrenal insufficiency. Although the function of the hypothalamo-hypophysial system is critical for patients who have undergone adrenal cortex autotransplantation, the details of that system, with the exception of adrenocorticotropic hormone in the subjects with adrenal autotransplantation, have been overlooked for a long time. To clarify the precise effect of adrenal autotransplantation on the pituitary gland and hypothalamus, the current study examined the gene expression of hormones produced from the hypothalamus and pituitary gland. Bilateral adrenalectomy and adrenal autotransplantation were performed in 8 to 9-week-old male rats. The hypothalamus and pituitary tissues were collected at 4 weeks after surgery. Transcriptional regulation of hypothalamic and pituitary hormones was subsequently examined by reverse transcription-quantitative polymerase chain reaction. Proopiomelanocortin, glycoprotein hormone α polypeptide, and thyroid stimulating hormone β were significantly elevated in the pituitary gland of autotransplanted rats when compared with sham-operated rats. In addition, there were significant differences in the levels of corticotropin releasing hormone receptor 1 (*Crhr1*), *Crhr2*, nuclear receptor subfamily 3 group C member 1 and thyrotropin releasing hormone receptor between the sham-operated rats and autotransplanted rats in the pituitary gland. In the hypothalamus, corticotropin releasing hormone and urocortin 2 mRNA was significantly upregulated in autotransplanted rats compared with sham-operated rats. The authors identified significant alterations in the function of not only the hypothalamus-pituitary-adrenal axis, but also the adenohypophysis thyrotropes in autotransplanted rats. In the future, it will be important to examine other tissues affected by glucocorticoids following adrenal cortex autotransplantation.

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Abbreviations: ACTH, adrenocorticotropic hormone; Atp5f1, ATP synthase H^+ transporting mitochondrial F_o complex subunit B1; Cga, glycoprotein hormones α polypeptide; Crh, corticotropin releasing hormone; Crhr1, corticotropin releasing hormone receptor 1; Crhr2, corticotropin releasing hormone receptor 2; Fshb, follicle stimulating hormone β polypeptide; Gh1, growth hormone 1; GRE, glucocorticoid responsive element; Hprt1, hypoxanthine phosphoribosyltransferase 1; Lhb, luteinizing hormone β polypeptide; Nr3c1, nuclear receptor subfamily 3 group C member 1; Prl, prolactin; Rplp2, ribosomal protein large P2; Trh, thyrotropin releasing hormone; Trhr, thyrotropin releasing hormone receptor; Tshb, thyroid stimulating hormone β; Ucn2, urocortin 2; Ywhaz; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein z; Pomc, proopiomelanocortin

Key words: autotransplantation, hypothalamo-hypophysial system, adrenal cortex, thyroid-stimulating hormone β

Introduction

The adrenal cortex comprises three layers: Zona glomerulosa, zona fasciculata and zona reticularis. The adrenal cortex mediates the stress response through the production of cortisol. Following bilateral adrenalectomy for the treatment of Cushing's disease, adrenocorticotropic hormone (ACTH)-independent macronodular adrenal hyperplasia and pheochromocytoma, cortisol replacement is necessary for the rest of patients' lives (1-4). However, patients experience side effects from long-term steroid treatment and are at risk of adrenal insufficiency. Autotransplantation of the adrenal cortex may be an alternative to steroid replacement therapy following bilateral pheochromocytoma, which is a form of catecholamine-producing neuroendocrine tumor (4). To avoid the side effects of cortisol replacement, autotransplantation following bilateral adrenalectomy is required. Successful, autotransplantation may lower the risk of adrenal insufficiency and improve the quality of life for patients.

Upregulation of glucocorticoids and ACTH levels in blood following autotransplantation has been reported in patients with pheochromocytomas following bilateral adrenalectomy (2,5).

Notably, there have not been any reports detailing the function of the hypothalamus and pituitary gland following adrenal autotransplantation. Because the autotransplanted adrenal gland does not have the full function of the original adrenal gland (6), dysfunction of the hypothalamus-pituitary axis may occur in patients following autotransplantation. However, the functional alterations in the hypothalamus and pituitary gland following autotransplantation are poorly understood. In the current study, the gene expression in the hypothalamus and pituitary were examined following adrenal autotransplantation using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis as a pilot study.

Materials and methods

Ethical approval. All experiments were conducted in accordance with the Guidelines (7) and were approved by the Ethics Committee on Animal Experiments of Kansai Medical University (Hirakata, Japan; approval ID: 15-002).

Animal preparation. A total of nine male Wistar rats (age, 8- to 9-weeks-old; weight, 180-240 g) were housed in sound-attenuated light-controlled cages (light on 8:00 a.m. and off 8:00 p.m.; 12 h light-dark cycle; constant environment at 25±1˚C and 50±10% relative humidity). Food and water were available *ad libitum*. Bilateral adrenalectomy was performed on 4 rats following laparotomy under general anesthesia by inhalation of 2% isoflurane (Pfizer Japan Inc., Tokyo, Japan) and 3 l/min oxygen. Resection of the adrenal medulla and midline and horizontal incision was conducted under a stereomicroscope. The 4 chopped-bilateral adrenal capsules and cortex with zona glomerulosa and undifferentiated cell zone (8) were autotransplanted in 4 rats in two abdominal muscle pockets that were formed by a pair of fine scissors (9). Tissue collections were performed at 4 weeks after autotransplantation between 9:00 a.m. and 11:00 a.m. (zeitgeber time (ZT) 1 to ZT3) in all animals. As a control, sham-operations without adrenalectomy were performed in 5 rats, and their tissues were also collected at 4 weeks after surgery. All animals received saline instead of water during the 10 days after surgery, because adrenal-autotransplanted rats cannot survive without saline for 10 days after surgery (10). The animals did not receive any steroid replacement, as rats can survive without steroid replacement following adrenalectomy (11). Rat hypothalamus was dissected coronally from the optic chiasma to the mammillary bodies (-6 mm from the chiasma) using a brain slicer (Zivic Instruments, Pittsburgh, PA, USA). The dorsal limit of the hypothalamus was the roof of the third ventricle, and the lateral limit was the amygdala (12). A total of 32 male Wistar rats (age, 11-12-weeks-old) were housed in same aforementioned conditions and decapitated to identify their diurnal variation in housekeeping genes at 4 h intervals over 24 h from ZT0 to ZT20 of the hypothalamus (n=4 for each ZT) or at ZT2 and ZT14 of the pituitary (n=4 for each ZT).

RT-qPCR. Total RNA was isolated from each individual hypothalamus and whole pituitary gland using Sepasol-RNA I Super G reagent (Nacalai Tesque, Inc., Kyoto, Japan). Single-stranded cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan). The expression level of each mRNA was determined by RT-qPCR with an ABI 7300 system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using the THUNDERBIRD qPCR mix (Toyobo Co., Ltd., Osaka, Japan) and gene-specific primers (Table I). PCR products were amplified using the following thermocycling conditions: 1 cycle, 1 min, 95˚C; 40 cycles, 10 sec, 95˚C, 60 sec, 60˚C.

The housekeeping gene with minimum diurnal variation was identified using a Rat Housekeeping Gene Primer set (Takara Bio, Inc.) in the hypothalamus at 4 h intervals over a 24 h period. Hypoxanthine phosphoribosyltransferase-1 (*Hprt1*), ribosomal protein large P2 (*Rplp2*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein-z (*Ywhaz*) primers were newly synthesized for the relative quantification of the gene expression in the hypothalamus and pituitary (Table I). Subsequently, the relative level of target gene expression was evaluated using the $2^{-\Delta\Delta Cq}$ method (13) with *Hprt1* as an internal control.

Statistical analysis. Distributions [mean ± standard deviation (SD)] of relative gene expressions were compared using unpaired Student's t-test in Microsoft Excel software. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Pituitary hormones and hypothalamic releasing hormones possess diurnal variation. In addition, certain housekeeping genes may also have this variation. Therefore, to avoid false-positive results caused by the sampling time and to increase the stringency of relative hormone mRNA measurements, the housekeeping genes with minimum diurnal variation were examined. The relative quantity of housekeeping gene expression was evaluated using the $2^{-\Delta\Delta Cq}$ method with ATP synthase H^+ transporting mitochondrial F_o complex subunit B1 used as the reference gene. The *Hprt1* gene had minimal variation over a 24 h period in the rat hypothalamus (mean ± SD; 1.08±0.05, coefficient of variation; 4.55%; Fig. 1; Table II). There was no significant difference in the relative expression of *Hprt1* in pituitary tissue between ZT2 (1.16±0.15) and ZT14 (1.18±0.20; P=0.865). *Hprt1* had minimal variation, when compared with *Rplp2* and *Ywhaz,* which were the housekeeping genes with the second and third lowest diurnal variation in the hypothalamus. In addition, there was no significant difference of *Rplp2* in the pituitary gland between ZT2 (1.37±0.26) and ZT14 (1.17±0.13; P=0.106) but, notably, there was a significant difference in the pituitary gland of *Ywhaz* between ZT2 (1.61±0.13) and ZT14 (1.09±0.09; P<0.001).

Subsequently, *Hprt1* was used as the internal control. Proopiomelanocortin (*Pomc*; 64.28±11.39 vs. 22.63±3.39; P<0.005), glycoprotein hormones α polypeptide (*Cga*; 1.69±0.14 vs. 1.16±0.17; P<0.01) and thyroid stimulating hormone β (*Tshb*; 9.60±3.61 vs. 3.90±1.02; P<0.05) were demonstrated to be significantly elevated in the pituitary gland of autotransplanted rats, when compared with sham-operated rats (Fig. 2). There was no significant difference in prolactin (*Prl*; 1.83±0.46 vs. 1.47±0.64), growth hormone-1 (*Gh1*; 2.06±1.53 vs. 1.89±0.77), luteinizing hormone β polypeptide (1.32±0.22 vs. 1.51±0.39), follicle stimulating hormone β

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Gene symbol	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
Gh1	NM_001034848.2	tgtttgccaatgctgtgctc	tgaatggaatagcgctgtcc
Prl	NM_012629.1	tttggtgcactcctggaatg	agccgcttgttttgttcctc
Tshb	NM_013116.2	ttccgtgcttttcgctcttg	agatggtggtgttgatggtcag
Fshb	NM_001007597.1	tgaagtcgatccagctttgc	atgcagaaacggcactcttc
Lhb	NM_012858.2	ttctgatgcccacccactaac	aagcctttattgggagggatgg
Cga	NM_053918.2	atcagtgtatgggctgttgc	atgatttggccacacagcac
Pomc	NM_139326.2	ttcatgacctccgagaagagc	tgtgcgcgttcttgatgatg
Crh	NM_031019.1	gaatacttcctccgcctggg	ggaaaaagttagccgcagcc
Crhr1	NM_030999.3	ttctgaacagtgaggtccgc	aggtggggatggacatagct
Crhr2	NM_022714.1	ccgaatcgccctcatcatca	ttcgtggtcgatgagttgca
Nr3c1 (GR)	NM_012576.2	aaatgggcaaaggcgatacc	agcaaagcagagcaggtttc
Trh	NM_013046.3	aaaagggcattgggtcatcc	acttgtgggctttgcttcac
Trhr	NM_013047.3	ccatcaacccggtgatttac	aaagcggtctgactccttga
Ucn2	NM_133385	atgtttctgaacccctcacg	gacacagctaggcacgacaa
Hprt1	NM_012583.2	cctgttgatgtggccagtaaag	atcaaaagggacgcagcaac
Rplp2	NM_001030021.1	attgaggatgtcatcgctcagg	tctttcttctcctctgctgcag
Ywhaz	NM_013011.3	ttcgcagccagaaagcaaag	ttgtcatcaccagcagcaac

Gh1, growth hormone 1; Prl, prolactin; Tshb, thyroid stimulating hormone β; Fshb, follicle stimulating hormone β polypeptide; Lhb, luteinizing hormone β polypeptide; Cga, glycoprotein hormones α polypeptide; Pomc, proopiomelanocortin; Crh, corticotropin releasing hormone; Crhr1, corticotropin releasing hormone receptor 1; Crhr2, corticotropin releasing hormone receptor 2; Nr3c1, nuclear receptor subfamily 3 group C member 1; Trh, thyrotropin releasing hormone; Trhr, thyrotropin releasing hormone receptor; Ucn2, urocortin 2; Hprt1, hypoxanthine phosphoribosyltransferase 1; Rplp2, ribosomal protein large P2; Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein-z.

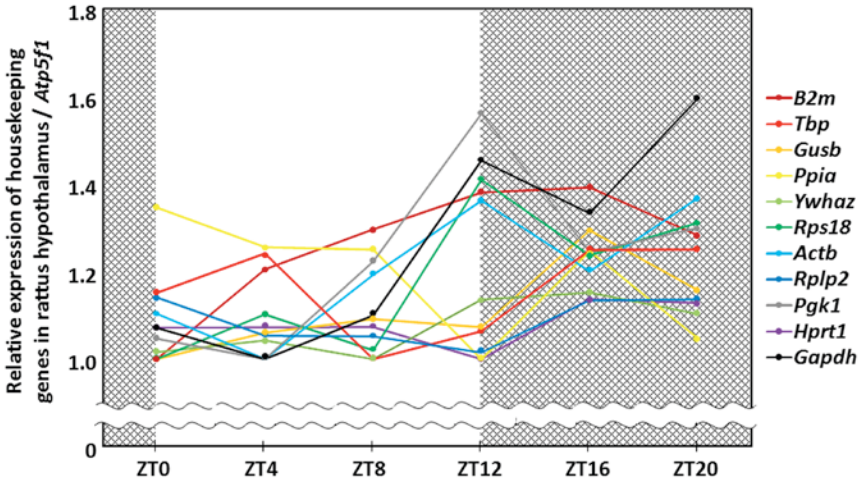

Figure 1. Diurnal rhythm of housekeeping gene expression in the rat hypothalamus. Each dot represents the mean value of relative expression of housekeeping genes at different ZTs. Atp5f1, ATP synthase, H^+ transporting, mitochondrial F_o complex subunit B1; B2m, β-2 microglobulin; Tbp, TATA box binding protein; Gusb, glucuronidase-β; Ppia, peptidylprolyl isomerase A; Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein z; Rps18, ribosomal protein S18; Actb, β-actin; Rplp2, ribosomal protein large P2; Pgk1, phosphoglycerate kinase 1; Hprt1, hypoxanthine phosphoribosyltransferase-1; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; ZT, zeitgeber time.

polypeptide (1.59±0.51 vs. 1.66±0.45) and urocortin-2 (*Ucn2*; 14.97±11.99 vs. 11.80±5.74) in the pituitary gland between sham-operated rats and autotransplanted rats (Figs. 2 and 3). There were significant differences in expression of corticotropin releasing hormone receptor 1 (*Crhr1*; 3.03±0.68 vs. 6.61±1.78; P<0.01), *Crhr2* (9.55±1.90 vs. 102.96±61.14; P<0.05), nuclear receptor subfamily 3 group C member 1 (*Nr3c1*; 7.86±7.81 vs. 63.35±34.86; P<0.05) and thyrotropin releasing hormone receptor (*Trhr*; 2.55±0.24 vs. 1.17±0.24; P<0.005) in the pituitary gland between sham-operated rats and autotransplanted rats (Fig. 3). In the hypothalamus, corticotropin releasing hormone (*Crh;* 1.65±0.55 vs. 2.45±0.31;

Table II. Diurnal variations of housekeeping genes in the rat hypothalamus.

Gene symbol	Mean expression[a]	Standard deviation	Coefficient of variation (%)
Hprt1	1.080	0.049	4.545
Rplp2	1.089	0.055	5.045
Ywhaz	1.075	0.064	5.940
Gusb	1.112	0.102	9.154
Tbp	1.159	0.107	9.250
Ppia	1.191	0.135	11.356
Actb	1.205	0.144	11.965
B2m	1.259	0.144	11.459
Rps18	1.180	0.165	14.007
Pgk1	1.229	0.201	16.313
Gapdh	1.259	0.239	18.953

[a]Mean expression across all time points (ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20) relative to ATP synthase H^+ transporting mitochondrial F_o complex subunit B1. Hprt1, hypoxanthine phosphoribosyltransferase 1; Rplp2, ribosomal protein large P2; Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein-z; Gusb, glucuronidase-β; Tbp, TATA box binding protein; Ppia, peptidylprolyl isomerase A; Actb, β-actin; B2m, β-2 microglobulin; Rps18, ribosomal protein S18; Pgk1, phosphoglycerate kinase 1; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

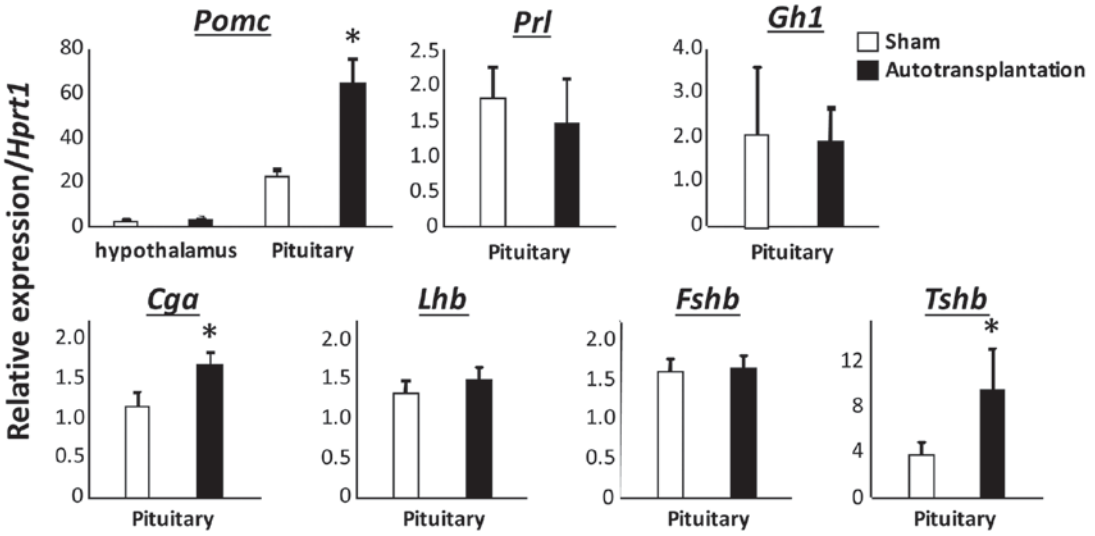

Figure 2. Relative expression of pituitary hormone mRNA transcripts. White bars and black bars indicate sham-operated and autotransplanted rats, respectively. Values are presented as the mean + standard deviations relative to *Hprt1*. *P<0.05 vs. sham-operated rats. *Hprt1*, hypoxanthine phosphoribosyltransferase 1; *Pomc*, proopiomelanocortin; *Prl*, prolactin; *Gh1*, growth hormone 1; *Cga*, glycoprotein hormones α polypeptide; *Lhb*, luteinizing hormone β polypeptide; *Fshb*, follicle stimulating hormone β polypeptide; *Tshb*, thyroid stimulating hormone β.

P<0.05) and *Ucn2* (150.03±127.97 vs. 611.46±252.98; P<0.01) were significantly upregulated in autotransplanted rats compared with sham-operated rats (Fig. 3). There were no significant differences in levels of *Pomc* (1.46±0.32 vs. 1.82±0.53), *Trh* (23.67±6.78 vs. 26.29±10.55), *Crhr1* (1.53±0.99 vs. 1.60±0.65), *Crhr2* (7.27±4.31 vs. 4.51±3.31) and *Nr3c1* (35.53±15.35 vs. 46.11±14.90) in the hypothalamus between sham-operated rats and autotransplanted rats (Fig. 3). In both the pituitary gland and the hypothalamus, there was no difference in *Rplp2* (1.48±0.52 vs. 1.46±0.15 in the hypothalamus; and 1.51±0.38 vs. 1.70±0.28 in the pituitary gland) and *Ywhaz* (2.34±0.67 vs. 2.70±0.21 in the hypothalamus; and 3.06±1.43 vs. 2.60±1.58 in the pituitary gland) between sham-operated rats and autotransplanted rats (Fig. 3).

Discussion

Autotransplantation following bilateral adrenalectomy helps to avoid steroid replacement therapy in postoperative pheochromocytoma patients. To the best of our knowledge, there are no studies regarding the hypothalamo-hypophysial system without ACTH in subjects following autotransplantation. To clarify the precise effect of adrenal autotransplantation on the pituitary and hypothalamic function, the authors examined whether there were significant differences in the hypothalamus-

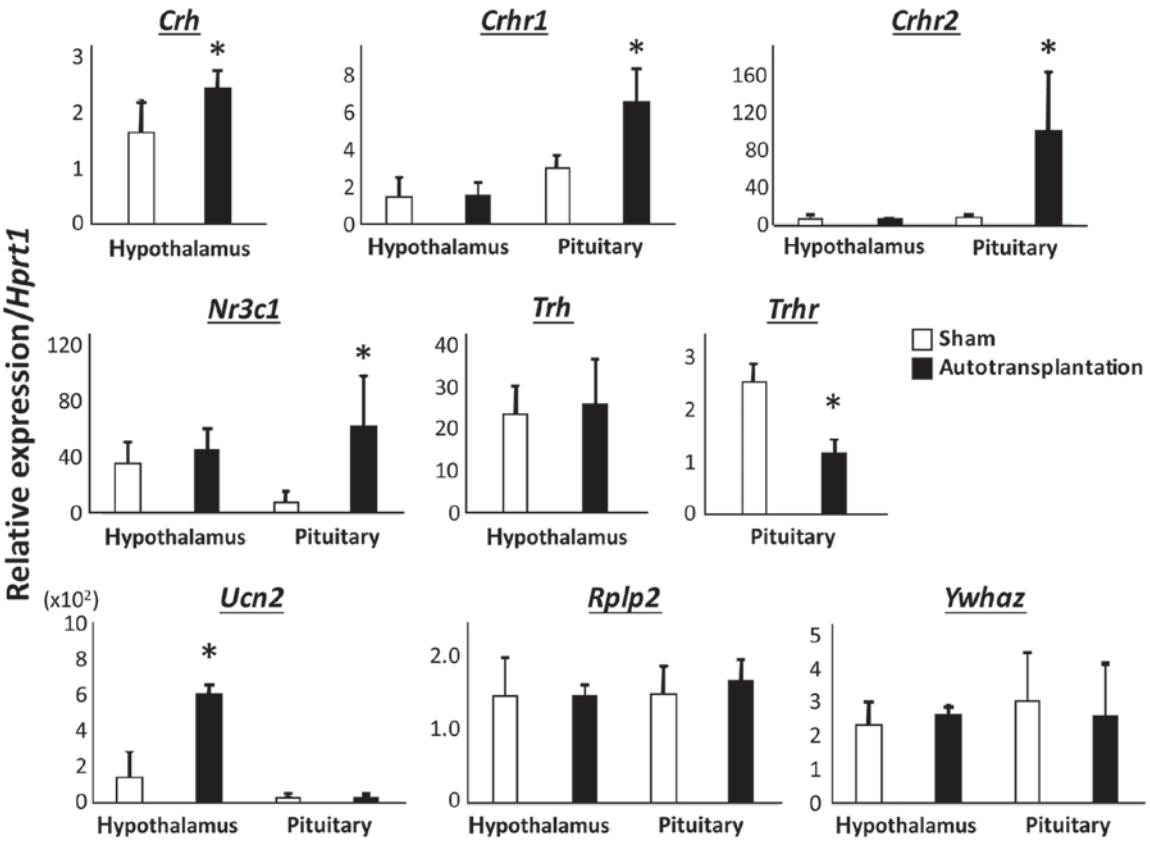

Figure 3. Relative gene expression in the hypothalamus and pituitary. White bars and black bars indicate sham-operated and autotransplanted rats, respectively. Values are presented as the mean ± standard deviations relative to *Hprt1*. *P<0.05 vs. sham-operated rats. *Hprt1*, hypoxanthine phosphoribosyltransferase 1; *Crh*, corticotropin releasing hormone; *Crhr1*, corticotropin releasing hormone receptor 1; *Crhr2*, corticotropin releasing hormone receptor 2; *Nr3c1*, nuclear receptor subfamily 3 group C member 1; *Trh*, thyrotropin releasing hormone; *Rplp2*, ribosomal protein large P2; *Ywhaz*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein z.

pituitary-adrenal axis, and other hormonal systems following adrenal autotransplantation. In the current study, there were increased levels of *Pomc*, *Cga*, *Tshb*, *Crhr1*, *Crhr2* and *Nr3c1* transcripts in the pituitary gland and *Crh* and *Ucn2* transcripts in the hypothalamus of autotransplanted rats compared with sham rats. In addition, the results demonstrated decreased levels of *Trhr* in the pituitary gland of autotransplanted rats compared with sham rats.

The hypothalamus neuropeptide, CRH is secreted from the paraventricular nucleus during stress responses. CRH activates the hypothalamic-pituitary-adrenal axis, modulating stress-induced ACTH secretion from the pars distalis. ACTH is proteolytically synthesized from the large precursor protein, POMC, by the anterior pituitary corticotrophs. The increase in blood ACTH level results in the adrenocortical release of cortisol and aldosterone (14,15). CRH itself is inhibited by glucocorticoids, which acts as a classical negative feedback loop. Therefore, the elevations of *Pomc*, *Crhr1*, *Crhr2*, *Nr3c1* and *Crh* transcripts in the present study are in line with the decrease in the negative feedback of glucocorticoids, due to the hypofunctioning autotransplanted adrenal cortex (6). The CRHR1 mediates the effects of CRH on the hypothalamus-pituitary-adrenal axis (16,17). The stress-inducible ACTH secretion from the anterior pituitary corticotrophs is impaired in *Crhr1*$^{-/-}$ mice (18,19). Hypersensitivity of the hypothalamus-pituitary-adrenal axis against stress conditions has been demonstrated in *Crhr2* null mice (20,21). One member of the CRH family, the UCN2 protein, selectively binds to CRHR2 (22) and an elevation of *Ucn2* mRNA was identified in the hypothalamus of the autotransplanted rats in the current study. Therefore, *Pomc* transcription in autotransplanted rats may be regulated by hypothalamic CRH and UCN2 in a coordinated manner.

In addition, *Cga* and *Tshb* expression are upregulated in the pituitary gland of autotransplanted rats. Glucocorticoids inhibit *Cga* expression mediated by the glucocorticoid responsive element (GRE) to the 5'-flanking region containing the cAMP-response element with a tissue-specific element of the *Cga* gene (23-25). Basal and thyrotropin-releasing hormone (TRH)-stimulated total TSH, CGA and TSHB secretion were decreased following dexamethasone administration in patients with hypothyroiditis (26). As chronic insufficiency of adrenocortical function due to autotransplantation induces low blood levels of glucocorticoids, it is speculated that subclinical hyperthyroiditis was induced in adrenal autotransplanted animals.

Tshb expression was upregulated in the pituitary gland of autotransplanted rats, however there is no report on the direct effect of glucocorticoid on *Tshb* transcription. The GRE in the upstream region of the *Tshb* gene has not yet been identified (27). By contrast, there is a GRE in the 5'-flanking region of the *Trh* gene. *Trh* transcription is directly regulated by glucocorticoids (28,29), therefore it was suggested that the increase in *Trh* expression and subsequent *Tshb* elevation

had occurred in the autotransplanted rats by the mechanism reported by Walter *et al* (30). Unexpectedly, there was no significant change in *Trh* expression in the current study. Prepro-TRH is synthesized in the neuronal cell bodies of various brain regions (31). Although several hypothalamic nuclei synthesize TRH, the TRH neurons regulating pituitary TSH release are localized exclusively to the paraventricular nucleus (32,33). In the present study, the whole hypothalamus was used to determine the expression level of *Trh*, therefore changes in *Trh* expression caused by autotransplantation in the paraventricular nucleus could not be detected in the present samples. Subsequently, the downregulation of *Trhr* expression was demonstrated to occur in the pituitary gland of the autotransplanted rats. The direct transcriptional enhancement of *Trhr* induced by glucocorticoids via GRE has been well described (34-36). These results suggested that *Tshb* expression in the pituitary gland of autotransplanted rats was regulated by a different pathway from the TRH-TRHR system or direct glucocorticoid effect.

In conclusion, the results identified an elevation in gene expression of the hypothalamus-pituitary-adrenal axis and adenohypophysis thyrotrophs in autotransplanted rats, suggesting that a small amount of cortisol replacement is required even following autotransplantion. Future studies will examine gene expression in other tissues following adrenal autotransplantation.

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