observed. The first case of a DOC-producing adrenocortical carcinoma was reported in 1974 [3], and a benign DOC-producing adrenocortical adenoma was reported in 1976 [4]. Overall, however, the total number of the report of adrenal tumor producing mineralocorticoid intermediates was not so large even now. Progesterone producing adrenal tumors are even rarer [5, 6]. Only two cases with progesterone producing adrenal tumors have been reported, and both presented with amenorrhea. This may in part reflect the fact that hypersecretion of steroid intermediates can often be overlooked, as these steroid intermediates are generally not measured in daily clinical practice. In our patient, steroid end products, including biologically active steroids, were within normal ranges, despite the elevated steroid intermediates. We therefore endeavored to account for this abnormal steroidogenesis by examining the urinary steroid profile and performing an immunohistochemical analysis of the steroidogenic enzymes in the resected adrenocortical tumor tissue.

Materials and methods

Steroid hormones in plasma were measured by SRL Co., Ltd. (Tokyo, Japan). The 2- and 8-mg dexamethasone suppression test was performed using the classical Liddle method. A detailed description of urinary steroid profile analysis is available in our earlier publication [7]. Briefly, 0.2–5 ml of urine was subjected to methyloxime-trimethylsilyl derivatization after enzymatic hydrolysis and organic solvent extraction. The derivative was subjected to gas chromatography/mass spectrometry-selected ion monitoring (GC/MS-SIM) analysis, and each steroid was identified based on its retention time and the ratio of its two characteristic mass ions, and quantified using stigmasterol as an internal standard (mg/g creatinine). The steroid contents of the tumor tissue were measured by Teikoku Hormone Medical Co., Ltd. (Kawasaki, Japan) using LC-MS/MS analysis. Immunohistochemical analyses of steroidogenic enzymes were performed as previously described [8, 9].

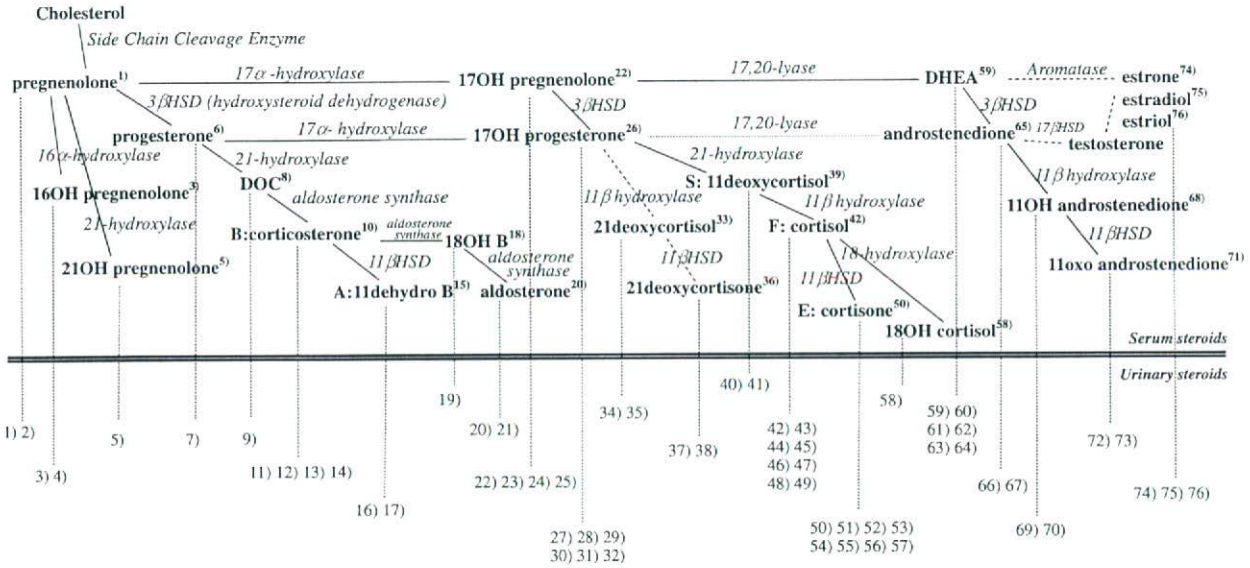

Fig. 1 Steroid metabolism map. *1* pregnenolone, *2* pregnenediol, *3* 16-hydroxypregnenolone, *4* 3,16,20-pregnenetriol, *5* 21-hydroxy-pregnenolone, *6* progesterone, *7* pregnanediol, *8* deoxycorticosterone (DOC), *9* tetrahydro-11-deoxycorticosterone, *10* corticosterone, *11* 5β-tetrahydrocorticosterone, *12* 5α-tetrahydrocorticosterone, *13* 20β-dihydrocorticosterone, *14* 6β-hydroxycorticosterone, *15* 11-dehydrocorticosterone, *16* 5β-tetrahydro-11-dehydrocorticosterone, *17* 5α-tetrahydro-11-dehydrocorticosterone, *18* 18-hydroxycorticosterone, *19* 18-hydroxy-tetrahydro-11-dehydrocorticosterone, *20* aldosterone, *21* tetrahydroaldosterone, *22* 17-hydroxypregnenolone, *23* 3,17,20-pregnenetriol, *24* 5,16-androstadiene, *25* 15,17-dihydroxypregnenolone, *26* 17-hydroxyprogesterone, *27* 5β-17-hydroxypregnanolone, *28* 5α-17-hydroxypregnanolone, *29* 20β-pregnanetriol, *30* 5β-20α-pregnanetriol, *31* 5α-20α-pregnanetriol, *32* 15,17-dihydroxypregnanolone, *33* 21deoxycortisol, *34* 11-hydroxy-17-hydroxypregnanolone, *35* pregnanetetrol, *36* 21-deoxycortisone, *37* 11-oxo-17-hydroxypregnanolone, *38* pregnanetriolone, *39* deoxycortisol, *40* 5β-tetrahydro-11-deoxycortisol, *41* 5α-tetrahydro-11-deoxycortisol, *42* cortisol, *43* 6β-hydroxycortisol, *44* 5β-tetrahydrocortisol, *45* 5α-tetrahydrocortisol, *46* 20α-cortol, *47* 20β-cortol, *48* 20α-dihydrocortisol, *49* 20β-dihydrocortisol, *50* cortisone, *51* 5β-tetrahydrocortisone, *52* 5α-tetrahydrocortisone, *53* 20α-cortolone, *54* 20β-cortolone, *55* 5α-20β-cortolone, *56* 20α-dihydrocortisone, *57* 20β-dihydrocortisone, *58* 18-hydroxycortisol, *59* DHEA, *60* androstenediol, *61* 16α-hydroxy-DHEA, *62* 16β-hydroxy-DHEA, *63* 16-oxo-androstenediol, *64* androstenetriol, *65* androstenedione, *66* androsterone, *67* etiocholanolone, *68* 11-hydroxyandrostenedion, *69* 11β-hydroxyandrosterone, *70* 11β-hydroxyetiocholanolone, *71* 11-oxoandrostenedione, *72* 11-oxoandrosterone, *73* 11-oxoetiocholanolone, *74* estrone, *75* estradiol, *76* estriol

Case report

A 27-year-old woman came to a hospital in April 2007 complaining of epigastric pain. Ultrasonography revealed a large tumor in her left adrenal region, and she was admitted to our hospital for further examination. She had been suffering from amenorrhea during the previous 2 years, though her menarche had been normal at 13 years of age. She was married but had no children. There was no family history of hypertension or endocrine tumors, and she had no past history. On physical examination, her height was 158.4 cm, body weight 45.4 kg, and body temperature 36.4°C. She denied having a recent weight change. Her blood pressure was 148/87 mmHg without postural change, and her pulse rate was 74 beats/min. Her lungs and heart were normal. The adrenal tumor was palpable under the left hypochondrium, and there were no clinical signs of virilization or hypercortisolism. Arterial blood gas analysis was nearly normal. An X-ray film of her chest was normal, and her ECG showed normal sinus rhythm but a U-wave.

The patient's laboratory results are summarized in Table 1. Serum potassium was 2.3 mEq/l, and plasma renin activity (PRA) was low (0.1 ng/ml/h), but plasma aldosterone was in the normal range (66 pg/ml). Plasma ACTH and serum cortisol were normal, and their diurnal rhythm was nearly normal (Table 2). The serum DOC level was markedly elevated and not suppressed by administration of 2- or 8-mg of dexamethasone (Table 3). Serum

Table 2 Diurnal rhythm of ACTH and cortisol

	8:00	12:00	16:00	20:00	23:00
ACTH (pg/ml)	36	24	16	9	8
Cortisol (μg/dl)	12.7	7.6	4.9	3.7	3.3

Table 1 Plasma biochemistry at 8:00 am before and after removal of the tumor

	Before	After		Plasma hormone	Before	After	
Electrolyte				ACTH	25	53	pg/ml (7–56)
Serum				LH	4.3	15.0	μU/ml (*1)
Na	143	140	mEq/l (136–144)	FSH	2.7	4.2	μU/ml (*2)
K	2.3	4.4	mEq/l (3.6–4.8)	PRL	9.1	21.8	ng/ml (6.12–30.54)
Cl	101	106	mEq/l (99–109)	Cortisol	10.4	12.6	μg/dl (5–15)
Urinary				PRA	0.1	0.8	ng/ml/h (0.2–2.7)
Na	104.6	94.5	mEq/day	Aldosterone	66	63	pg/ml (30–159)
K	35.7	24	mEq/day	DOC	8.04	0.09	ng/ml (0.03–0.33)
Cl	117.3	94.5	mEq/day	Corticosterone	5.88	–	ng/ml (0.21–8.43)
Renal function				18-OH DOC	0.13	0.06	ng/ml (0.01–0.07)
BUN	8	15	mg/dl (8–22)	Progesterone	3.53	0.52	ng/ml (*3)
Creatinine	0.5	0.9	mg/dl (0.4–0.8)	17-OH prog	3.0	0.8	ng/ml (0.2–2.8)
Urinary hormone				Pregnenolone	5.04	0.31	ng/ml (0.2–1.5)
17-OHCS	4.8	5.1	mg/day (2.4–11.8)	Estradiol	34.2	32.0	pg/ml (*4)
17-KS	6.9	6.1	mg/day (2.2–7.3)	DHEA-S	80	95	ng/ml (73–322)
Adrenaline	2.1	4.0	μg/day (3.4–26.9)	Testosterone	48.0	30.1	ng/dl (9.12–111)
Noradrenaline	48.0	235.4	μg/day (48.6–168.4)	Adrenaline	9	14	pg/ml (<100)
Dopamine	808.5	2181	μg/day (365.0–961.5)	Noradrenaline	125	117	pg/ml (100–450)
Metanephrine	0.03	0.05	mg/day (0.04–0.19)	Dopamine	5	9	pg/ml (<20)
Normetanephrine	0.10	0.14	mg/day (0.09–0.33)				

	Follicular phase	Ovulation	Luteal phase	Menopause
*1	1.76–10.24	2.19–88.33	1.13–14.22	5.72–64.31
*2	3.01–14.72	3.21–16.60	1.47–8.49	<157.79
*3	<0.92	<2.36	1.28–29.6	<0.44
*4	20–350	50–550	45–300	<21

Values in parentheses indicate normal range

BUN blood urea nitrogen, *17-OHCS* 17-hydroxycorticosteroids, *17-KS* 17-ketosteroids, *ACTH* adrenocorticotropic hormone, *LH* Lutenizing hormone, *FSH* follicle stimulating hormone, *PRL* prolactin, *PRA* plasma renin activity, *DOC* 11-deoxycorticosterone, *18-OH DOC* 18-hydroxydeoxycorticosterone, *17-OH prog* 17-hydroxyprogesterone, *DHEA-S* dehydroepiandrosterone sulfate

Table 3 Dexamethasone suppression test (Liddle method)

	Base data	2 mg day 1	2 mg day 2	8 mg day 1	8 mg day 2	Normal range
Serum						
ACTH (pg/ml)	25	<5	<5	<5	<5	7–56
Cortisol (μg/dl)	10.4	3.6	4.0	3.8	4.3	5–15
DOC (ng/ml)	8.04	6.97	6.72	6.52	5.77	0.03–0.33
18-OH DOC (ng/ml)	0.13	0.17	0.18	0.19	0.16	0.01–0.07
Progesterone (ng/ml)	3.53	3.99	3.86	4.13	4.79	<0.92
Pregnenolone (ng/ml)	5.04	4.63	4.63	4.26	5.88	0.2–1.5
Urine						
17-OHCS (mg/day)	4.8	5.3	5.0	5.9	7.0	2.2–7.3
17-KS (mg/day)	6.9	5.9	4.2	3.9	3.8	2.4–11.0
Free cortisol (μg/day)	23.1	26.7	8.9	9.2	18.1	11.2–80.3

18-hydroxydeoxycorticosterone (18-OH-DOC) was also high, though serum corticosterone (B) was within the normal range. Serum pregnenolone, progesterone, and 17-OH progesterone were also high, but testosterone and dehydro-epi-androsterone-sulfate (DHEA-S) were in the normal range. Serum and urinary concentrations of catecholamines and their metabolites were all normal (Table 1).

We also performed a complete analysis of the patient's urinary steroid metabolites (Fig. 1 and Table 4). Her urinary steroid profile showed elevated secretion of 17-deoxysteroids or 11-deoxysteroids (progesterone, DOC, 11-dehydrocorticosterone (data not shown), and 11-deoxycortisol), as well as 3β-hydroxy 5-en steroids (pregnenolone, 17-OHpregnenolone, and DHEA). Moreover, these metabolites were not suppressed by administration of dexamethasone (Table 4).

Computed tomography revealed a heterogeneous tumor in the left adrenal region (12 cm), which was pushing down on the left kidney (Fig. 2a, b). Magnetic resonance imaging (MRI) also revealed a heterogeneous tumor, which was of partially high density on T2 emphasizing phase images (data not shown). F-fluorodeoxyglucose positron emission tomography showed strong accumulation in the left adrenal mass, but no abnormal accumulation in other organs (Fig. 2c).

After a diagnosis of an adrenal tumor producing DOC and progesterone, the tumor was resected through a posterolateral skin incision. The tumor was 17 × 11 × 7.8 cm in size and weighed 480 g; the cut surface was yellowish brown in color and solid.

Based on the Weiss criteria, the resected tumor was diagnosed as an adrenocortical carcinoma. Nuclear grade was III, mitotic rate was >5 per 50 HPF, atypical mitosis was absent, character of cytoplasm was unclear, architecture of tumor was diffuse, necrosis was present, invasion of capsule was present, invasion of sinusoidal structure was present, and invasion of venous structure was absent. More than 10% of the cells were MiB 1 (Ki67)-positive (data not shown).

Table 5 shows the steroid contents in the tumor tissue. In comparison with the concentration in serum, the contents of pregnenolone, progesterone, and DOC in the tumor tissue were extremely high. This result suggests this tumor had produced these steroids.

Analysis for steroidogenic enzymes demonstrated that P450SCC immunoreactivity was diffusely positive in carcinoma cells (Fig. 3a). P450c17 immunoreactivity was not detected in the great majority of carcinoma cells (Fig. 3b). 3β-Hydroxysteroid dehydrogenase (3β-HSD) immunoreactivity was very sparsely detected (Fig. 3c). P450c21 immunoreactivity was sporadically detected (Fig. 3d). P450c11 immunoreactivity was detected in some tumor cells (data not shown).

Postoperatively, the patient's blood pressure dropped to about 100/60 mmHg, and her serum potassium level normalized without medication. Menorrhea occurred 3 months after the tumor resection. The postoperative endocrinological findings are shown in Table 1. Serum concentrations of DOC and progesterone returned to normal after the operation (Table 1), as did urinary levels of steroid metabolites (Table 4).

Discussion

We report here a patient with hypertension, hypokalemia, and amenorrhea accompanying an adrenal tumor. Because hypokalemia and low plasma renin activity were observed, despite normal plasma aldosterone levels, we presumed that the left adrenal tumor was producing mineralocorticoid intermediates such as DOC or corticosterone. Hypokalemia is most often caused by increased potassium elimination from the kidney or intestine, by insufficient oral uptake or by intracellular uptake of potassium. Our patient had taken

Table 4 Urine steroid metabolites data (mg/g creatinine)

Origin steroid	Metabolites no. in Fig. 1	Pre-Dex	Dex 2 mg	Dex 8 mg	Post-OPE	Normal range of adult female (18–49 years)			
						N	5 percentile	50 percentile	95 percentile
Pregnenolone	Σ1,2	16.800 H	12.684 H	14.925 H	0.452 H	189	0.000	0.000	0.083
Progesterone	7	11.460 H	14.994 H	18.538 H	0.484	189	0.169	0.490	3.236
DOC	9	2.467 H	2.336 H	2.386 H	0.007	187	0.000	0.000	0.040
Corticosterone	Σ11,12,16,17	1.345 H	0.904	0.964	0.285 L	189	0.307	0.600	1.067
18OHcorticosterone	19	0.023	0.009 L	0.015 L	0.017 L	63	0.020	0.043	0.117
Aldosterone	21	0.002 L	0.000 L	0.000 L	0.002 L	115	0.010	0.028	0.061
17OHpregnenolone	Σ22,23	2.757 H	1.715 H	2.301 H	0.244	189	0.121	0.250	0.554
17OHprogesterone	Σ27–32	1.263	1.176	1.320	0.459	188	0.321	0.680	1.644
11deoxycortisol	40	2.915 H	2.502 H	3.249 H	0.055	186	0.030	0.065	0.151
Cortisol	Σ44,45,51,52	6.824	1.879 L	2.051 L	4.862	189	3.552	5.430	8.178
DHEA	Σ59–64	6.026 H	1.472	1.384	1.810	189	0.434	1.390	4.076
Androstenedione	Σ66,67	1.747	0.734 L	0.772 L	2.713	189	1.372	2.500	4.292
11OH androstenedione	69	0.884	0.232 L	0.259 L	0.617	189	0.332	0.590	0.966
Estrogen	Σ74–76	0.031	0.025	0.029	0.010	189	0.000	0.025	0.081

no drug, had been eating a normal diet, and had no diarrhea. On the other hand, her urinary elimination of potassium was more than 35 mEq/day despite her low serum potassium (2.3 mEq/l), and her TTKG was 7.27. This patient's hypokalemia thus appeared to have been the result of renal potassium loss. Moreover, our finding that the patient's serum and urinary potassium normalized after resection of her tumor eliminated the possibility that a kidney disease such as renal tubular acidosis was the cause, further confirming that the hypokalemia was caused by the adrenal tumor. On the other hand, the serum and urinary levels of several adrenal steroid intermediates were elevated in this patient. The adrenal mineralocorticoids that can cause renal potassium loss are aldosterone, cortisol, corticosterone, and DOC. In this patient, basal serum aldosterone, cortisol, and corticosterone were nearly in the normal range, but DOC was extraordinarily high. Her 18-OH DOC was also slightly higher than normal, but 18-OH DOC has no effect on urinary potassium excretion, though it does stimulate hydrogen ion excretion [10]. The high serum DOC level normalized after the tumor was resected, along with the serum potassium level. In addition, the DOC content in the resected tumor tissue was high in comparison to the concentration in serum. The mineralocorticoid activity of DOC is about one twentieth that of aldosterone. This patient's serum DOC concentration was 8.04 ng/ml, which corresponds to the activity of about 400 pg/ml aldosterone, which is consistent with her serum and urinary potassium levels. Therefore, we diagnosed hypersecretion of DOC from an adrenal tumor as the major cause of our patient's hypokalemia.

In addition to her hypokalemia, this patient also exhibited amenorrhea that was cured after removal of her tumor. Amenorrhea with an adrenal tumor is most often related to hypersecretion of androgens. In this case, however, biologically active androgens were not elevated. We therefore speculated that the hypersecretion of progesterone and related hormones was the cause of the patient's amenorrhea. A connection between amenorrhea and progesterone has been reported in congenital adrenal hyperplasia characterized by 21-hydroxylase or 17-hydroxylase deficiency [11, 12]. Amenorrhea in patients with congenital adrenal hyperplasia is attributable in part to high progestogenic steroid levels exerting a "mini-pill (progestin contraceptive)" effect on the endometrium [13]. Progestin contraceptives are known to dose-dependently suppress FSH levels and reduce both the number and amplitude of LH pulses [14]. However, the minimal dose of progesterone needed to cause menstrual cycling to cease is not well established. In this case, FSH and LH were not completely suppressed, but their levels were below or near the lower limit of the normal range. This might be compatible with the patient's serum progesterone level, which was

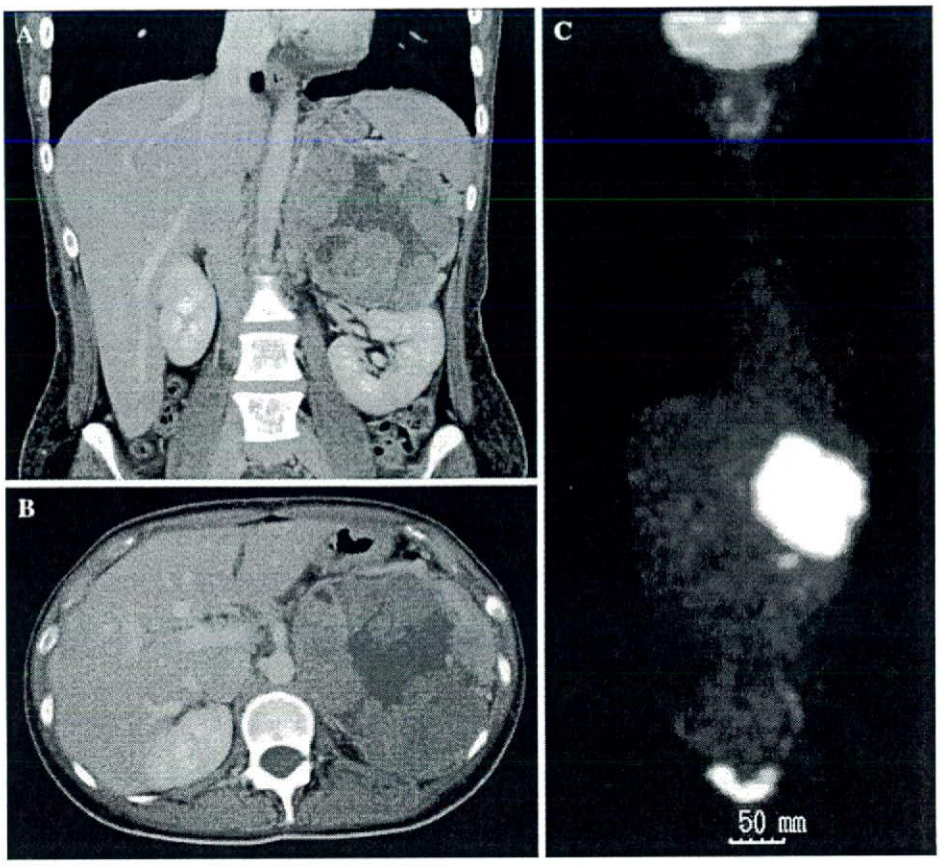

Fig. 2 **a** Contrast enhanced computed tomography, *coronal image*. **b** Contrast enhanced computed tomography, *horizontal image*. **c** F-fluorodeoxyglucose positron emission tomography

Table 5 Steroid contents in tumor tissue

Pregnenolone (ng/g)	1533.1
Progesterone (ng/g)	830.0
17OH pregnenolone (ng/g)	42.5
17OH progesterone (ng/g)	78.1
DOC (ng/g)	141.7
Cortisol (ng/g)	563.3
Aldosterone (pg/g)	83.5
DHEA (ng/g)	97.8

continuously higher than the normal range for the follicular phase, but not markedly so. Her urinary pregnanediol, which is the metabolite of progesterone, was 11.46 (mg/g creatinine) and higher than the normal range for the Luteal phase (min 1.2 to max 6.9 mg/g creatinine, $n = 26$). This suggests continuous high production of progesterone. We measured FSH and LH only once prior to the patient's surgery, but repeated measurements would have been better for determination of her LH level. Another possible explanation for the secondary amenorrhea in this patient is hypothalamic amenorrhea caused by the carcinoma. However, as the general condition of this patient was good and no weight loss was observed, we suggest it is unlikely that hypothalamic amenorrhea contributed significantly to her amenorrhea.

We also examined the ACTH dependency of this hypersecretion of steroid intermediates and found that the secretion was not suppressed during the dexamethasone suppression test (Table 3). This finding is consistent with earlier reports indicating that plasma DOC levels are under the control of ACTH in benign DOC-producing tumors, but not in malignant tumors [3, 4, 15–17]. Dexamethasone suppresses adrenal steroidogenesis by suppressing ACTH secretion from the pituitary gland. ACTH is known to regulate quantitatively the steroidogenesis at the first step, the conversion of cholesterol to pregnenolone, which is the enzymatically rate-limiting step [18]. Chronic regulation is principally at the level of transcription of the gene for cholesterol side-chain cleavage P-450 (P450SCC). Acute regulation is mediated by steroidogenic acute regulatory protein, which facilitates the rapid influx of cholesterol into mitochondria, where P450SCC resides [18]. Our result suggests that this regulatory system would be disordered in this adrenocortical carcinoma cells.

Pregnenolone, progesterone, and DOC were also not suppressed in the dexamethasone suppression test, but cortisol was suppressed (Table 3). This suggests that

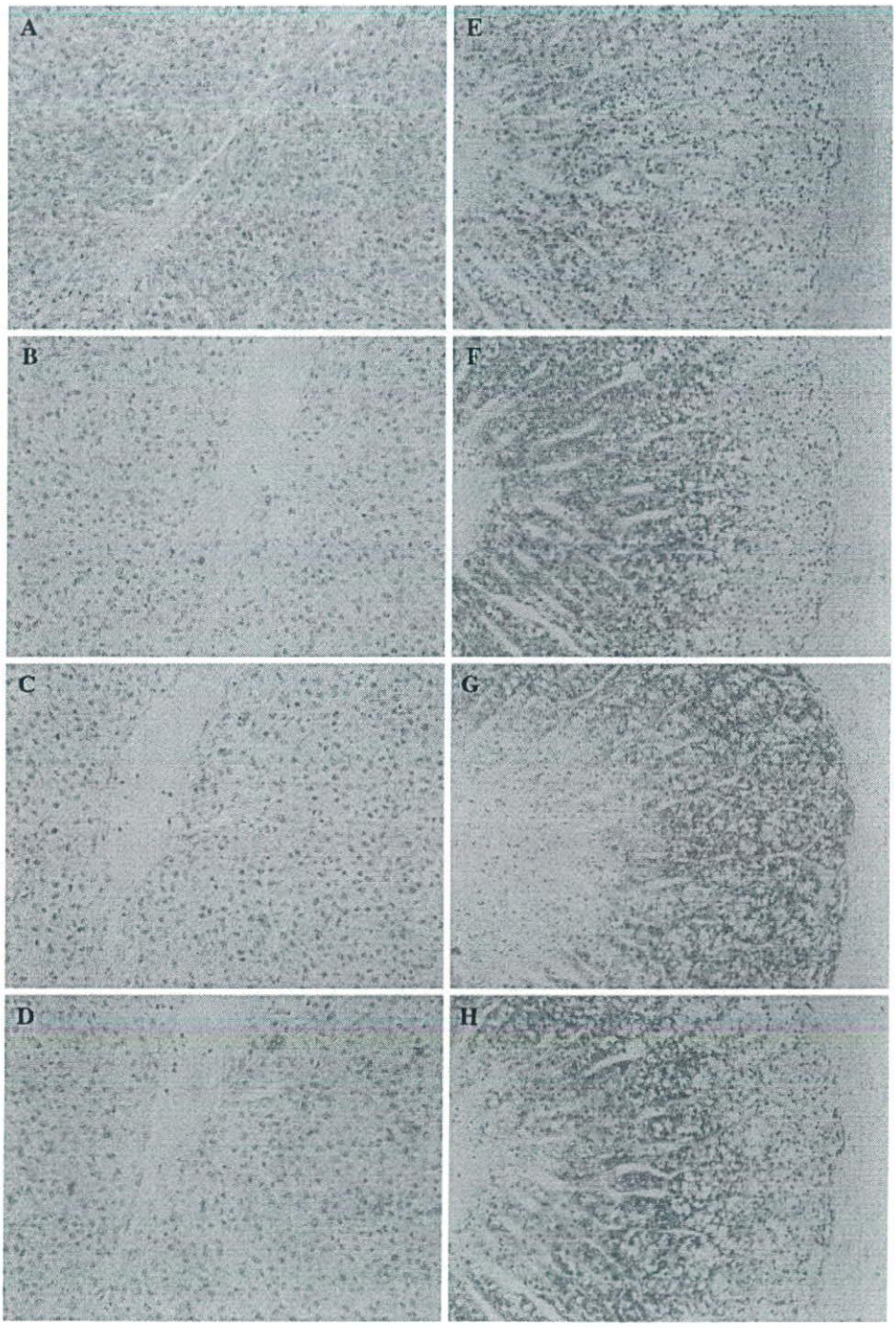

Fig. 3 Immunohistochemical analysis of steroidogenic enzymes. **a–d** The adrenocortical carcinoma, **e–h** a normal adrenal gland in another patient (*right*: zona gromerulosa). **a**, **e** P450SCC. **b**, **f** P450c17. **c**, **g** 3β-hydroxysteroid dehydrogenase (3β-HSD). **d**, **h** P450c21

pregnenolone, progesterone, and DOC were autonomously secreted from our patient's adrenal tumor, whereas cortisol was secreted from the normal adrenal gland (Fig. 1). The reason why not cortisol but pregnenolone, progesterone, and DOC were secreted from the tumor would be the deficiency of P450c17 and P450c11 in the tumor cells. It has been reported that levels of the transcriptional factor SF-1 are low, while those of nuclear receptor DAX-1 are high, in DOC-producing adrenal tumors [19]. As a consequence, expression of the steroidogenic enzyme P450c17 is weak [19]. Cytochrome P450c17 catalyzes both 17α-hydroxylation and 17,20-lyase conversion of 21-carbon steroids to 19-carbon precursors of sex steroids [20]. In the absence of P450c17, steroidogenic cells produce C21 17-deoxysteroids [18]. It also has been suggested that expression of steroidogenic enzymes plays a key causative role in the

Table 6 Ratios of urine steroid metabolites data

Enzyme activity	Origin steroid ratio	Metabolites no. in Fig. 1	Pre-Dex	Dex 2 mg	Dex 8 mg	Post-OPE	Normal range of adult female (18–49 years)			
							N	5 percentile	50 percentile	95 percentile
3βHSD	Progesterone/pregnenolone	7/2	0.06 L	0.05 L	0.05 L	2.00 L	65	2.16	5.92	15.89
	Androstenedione/DHEA	Σ66,67/Σ59–64	0.29 L	0.50 L	0.56 L	1.50	189	0.61	1.80	4.60
17α-hydroxylase	17OHpregnenolone/pregnenolone	23/2	0.46 L	0.41 L	0.45 L	5.98	65	2.03	4.72	16.82
	17OHprogesterone/progesterone	30/7	0.08 L	0.06 L	0.05 L	0.68	189	0.23	0.86	1.68
21-hydroxylase	DOC/progesterone	9/7	0.22 H	0.16 H	0.13 H	0.01	188	0.00	0.00	0.05
	11deoxycortisol/17OHprogesterone	40/30	3.33 H	2.96 H	3.48 H	0.17	189	0.04	0.16	0.44
11β-hydroxylase	Corticosterone/DOC	Σ11,12,16,17/9	0.55 L	0.39 L	0.40 L	40.71	85	10.56	51.00	155.01
	Cortisol/11deoxycortisol	Σ44,45,51,52/40	2.34 L	0.75 L	0.63 L	88.40	185	36.56	80.80	164.60

pathophysiology stemming from steroid-producing adrenal tumors [21, 22].

In the present case, the ratios of 17α-hydroxysteroids to 17-deoxysteroids (17OHpregnenolone/pregnenolone and 17OHP/progesterone), 11β-hydroxysteroids to 11-deoxysteroids (corticosterone/DOC and cortisol/11-deoxycortisol), Δ4 steroids to 3β-hydroxy 5en steroids (progesterone/pregnenolone and androstenedione/DHEA) were decreased, and the ratios of 21-hydroxysteroids to 21-deoxysteroids (DOC to progesterone and 11-deoxycortisol to 17OHP) increased, compared with control females. The abnormality of these metabolite ratios suggests impairment of 17α-hydroxylase, 11β-hydroxylase and 3β-HSD activities, and enhanced 21-hydroxylase activity in the tumor tissue (Fig. 1 and Table 6). On immunohistochemical analysis of the steroidogenic enzymes, P450SCC immunoreactivity was highly detected in the great majority of carcinoma cells, while P450c17 and 3β-HSD immunoreactivity was little detected. P450c21 and P450c11 immunoreactivity was detected in some of the carcinoma cells. These results are compatible with that of the analysis of urinary steroids.

Our analyses also revealed heterogeneity of the steroidogenesis in this adrenocortical carcinoma, which was accompanied by a corresponding heterogeneity in the expression of steroidogenic enzymes termed "disorganized steroidogenesis" in an earlier report on adrenocortical carcinomas [8]. In adrenocortical carcinomas, disordered expression of steroidogenic enzymes within tumor tissues can lead to hypersecretion of various steroid intermediates. Notably, however, this can often be overlooked because these intermediates are generally not measured in routine clinical practice. Thus, the presence of an adrenal tumor that produces steroid intermediates should not be overlooked in the differential diagnosis of hypokalemia or amenorrhea.

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Orexin decreases mRNA expressions of NMDA and AMPA receptor subunits in rat primary neuron cultures

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ABSTRACT

Orexin is one of the orexigenic neuropeptides in the hypothalamus. Orexin neurons in the lateral hypothalamus (LH) project into the cerebral cortex and hippocampus in which the receptors are distributed in high concentrations. Therefore, to elucidate the actions of orexin in the cerebral cortex, we examined its effects on the mRNA expressions of *N*-methyl-D-aspartate (NMDA) receptor subunits (NR1, NR2A, NR2B) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor subunits (GluR1, GluR2) following 6-day application of orexin-A or orexin-B to rat primary cortical neuron cultures. The mRNAs of NR1 and NR2A subunits were significantly decreased by orexin-A and orexin-B at concentrations over 0.1 μM and 0.01 μM, respectively. The mRNA expression of NR2B subunit was also significantly decreased by orexin-A and orexin-B only at the concentration of 1 μM. Moreover, orexin-A and orexin-B at concentrations over 0.01 μM significantly decreased the mRNA expressions of AMPA receptor subunits, GluR1 and GluR2. The present study demonstrated that orexins significantly suppressed RNA expressions of NMDA and AMPA receptor subunits in cortical neuron cultures, suggesting that orexin may regulate the higher functions of the cerebral cortex as well as be involved in energy regulation in the hypothalamus.

1. Introduction

The hypothalamus is a center of energy regulation containing orexigenic and anorexigenic neuropeptides, and its dysregulation is thought to induce obesity [36]. Obesity is a risk factor for metabolic diseases, such as type 2 diabetes mellitus, hypertension and hyperlipidemia [26]. Among these neuropeptides, orexins (orexin-A, orexin-B) containing neurons were observed to be present in the lateral hypothalamus (LH) with projections into the cerebral cortex, hippocampus and amygdala [13,30], which are the essential regions for controlling cognition [21], anxiety and depression [24]. Orexins in LH were reported to be up-regulated in obese rodent models [28]. The actions of orexins are mediated via two receptors, orexin-1 (OX1R), and orexin-2 (OX2R), that are coupled with G-proteins [34]. While

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orexin-A has equal affinity for OX1R and OX2R, orexin-B has an appreciably greater (approximately 10-fold) affinity for OX2R [34]. In situ hybridization studies have shown that high levels of orexin-1 receptor and orexin-2 receptor mRNA occur in the cerebral cortex [15,21]. Endogenous orexins have diverse physiological functions related to food intake [34], arousal [13], sleep-waking cycle [31,41], sleep disorder [22], nociception [7], and learning and memory [1,2,16,20,40], anxiety and depression [24].

In the cerebral cortex, the ionotropic glutamatergic nervous system, including N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) glutamate receptors, plays a crucial role in the regulation of learning and memory within cortico-hypothaoamic networks [4,5,8]. The NMDA receptors are composed of NR1, NR2A-D, and NR3 subunits, with the NR1 subunit being a necessary component of functional NMDA receptor channels [10,29,33]. The AMPA receptors composed of GluR1-4 subunits are distributed in the cerebral cortex, and also play an important role in neural plasticity related to learning and memory with interaction NMDA receptor signaling [6,35]. It was reported that depletion of NR2A subunit of NMDA receptor in mice induced memory dysfunction [23], and that depletion of NR2B subunit of NMDA receptor in mice impairment of spatial learning [12]. Intracerebroventricular administrations of AMPA receptor antagonists CNQX and NBQX, were reported to induce impairment of memory [6,35]. Orexin was demonstrated to increase glutamatergic activity in several brain areas innervated by orexin neuronal fibers, including the LH/PFA [14] Glutamate and orexin are colocalized within fibers in the tuberomamillary nucleus [14] and hypothalamus [14], and the postsynaptic action of orexin to stimulate pyramidal cells in the prefrontal cortex was facilitated by glutamate in a synergistic fashion [14]. In addition, glutamate release was increased in response to systemic administration of orexin-A in both the locus coeruleus and amygdala [14].

Recent studies have shown that central administration of orexin-A and orexin-B has effects on learning and memory but the literature concerning the role of the orexin system in cognition remains controversial. Aou et al. have reported that i.c.v. administered orexin-A produced memory impairment in water maze performance in a 2-day training protocol [2]. On the contrary, Akbari et al. reported that the intra-CA1 injection of the selective OX1R antagonist impaired acquisition, consolidation and retrival of spatial memory in Morris water maze task [1]. They also reported that the intra-dentate gyrus administration of OX1R antagonist impaired acquisition and consolidation of Morris water maze task, but had no effect on retrieval in spatial memory [1].

We postulated that the ionotropic glutamatergic nervous system in the cerebral cortex may be modulated by orexins. To test this, we used the primary cortical neuronal culture, which is well understood and an established method for examining the physiological effect on adult brain plasticity of a specific substance on the ionotropic glutamatergic nervous system [38]. In the present study, we examined the mRNA expressions of NMDA and AMPA receptor subunits after chronic application of orexin-A and orexin-B by using rat cultured primary cortical neurons.

2. Materials and methods

2.1. *Rat cultured primary cortical neurons*

Timed pregnant Sprague–Dawley rats were obtained from Japan SLC, Inc. (Japan) on gestational day 18. The animals were anesthetized with pentobarbital sodium (50 mg/kg, ip; Abbott, Abbott Park, Ill, USA) and sacrificed by cervical dislocation. The fetuses were delivered and decapitated. For each experiment, fetuses were extracted from four maternal rats. All experiments were performed in accordance with the guidelines established by the Institutional Animal Investigation Committee at Kyoto University, Chiba University and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to optimize the comfort and to minimize the use of animals. The cultured primary cortical neurons were prepared according to previous reports [9,18,19] with minor changes. The cerebral cortex was dissected and cut into small pieces in Hanks' balanced salt solution (HBSS: Ca^{2+} and Mg^{2+} free) (Invitrogen, Carlsbad, CA, USA). The tissue was then dispersed with 0.025% trypsin-EDTA solutions (Invitrogen, Carlsbad, CA USA). DNase (final concentration; 0.2 μg/ml) (Sigma, St. Louis, MO, USA), soybean trypsin inhibitor (final concentration; 2 μg/ml) (Sigma, St. Louis, MO, USA), $MgSO_4$ (final concentration; 0.24 mM) were added. The cell suspension was centrifuged at 800 × *g* for 5 min, and the supernatant was aspirated. The cells were suspended in Neurobasal Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2% of B27 containing antioxidants (Invitrogen, Carlsbad, CA, USA), L-glutamine (final concentration; 0.5 mM), antibiotic–antimycotic solution (final concentration; 1%) (Nacalai tesque, Kyoto, Japan), then DNase (final concentration; 0.2 μg/ml) (Sigma, St. Louis, MO, USA), soybean trypsin inhibitor (final concentration; 2 μg/ml) (Sigma, St. Louis, MO, USA) and $MgSO_4$ (final concentration; 0.24 mM). The cell suspension was centrifuged at 800 × *g* for 5 min, the supernatant was aspirated, and the remainder was washed with Neurobasal Medium. This procedure was repeated 3 times. Live cells were counted using a hemocytometer, and the cell suspension was then diluted with Neurobasal Medium at 2×10^6 cells/ml. Cells were seeded onto poly-D-lysine-coated 6-well plates (BD Bioscience, Discovery Lab ware, Bedford, MA, USA) at 2×10^6 cells/ml well. All cultures were maintained in Neurobasal medium at 37 °C in 95% humidified air and 5% CO_2. On the 3rd day of culture, cytosine β-D-arabino-furanoside hydrochloride (final concentration; 10 μM) (Sigma, St. Louis, MO, USA), a selective inhibitor of DNA synthesis, was added for 72 h to the culture to prevent further proliferation of non-neuronal cells.

Orexin-A and orexin-B (0.01 μM, 0.1 μM and 1 μM) were applied to the culture wells on the 6th day of culture, and the cultured cells were incubated for 6 days. The culture medium which contained orexin-A, orexin-B or vehicle was changed every 3 days. On the 12th day of culture, the total cellular RNA was extracted from two cultured wells as one RNA sample using an RNeasy Mini Kit (Quiagen Sciences, MA, USA). The samples were stored at −20 °C until assay. All experiments consisted of 3–6 repetitive runs. For each run, two to six mRNA samples were obtained. Orexin-A and orexin-B were from

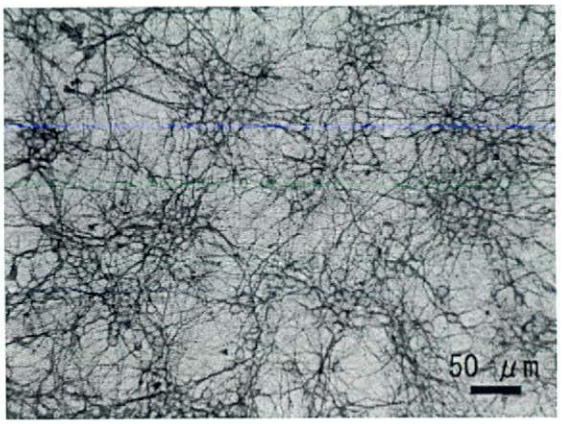

Fig. 1 – Rat cultured cortical neurons stained with anti-MAP2 antibody.

Peptide Institute Inc. (Osaka, Japan). Neuronal degeneration was assessed every 3 days after neuropeptide application by efflux of lactate dehydrogenase using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Co., Madison, WI, USA) [19].

2.2. *Immunohistochemical method*

For verification of cultured cortical neurons, expression of microtubule-associated protein-2 (MAP-2) predominantly in the dendrites of neurons was observed (Fig. 1). Cortical neuron cultures were prepared on D-poly-L-lysine-coating slide glasses (Matsunami, Osaka, Japan) according to the above noticed method. Cultured neurons at day 6th were fixed with 4% paraformaldehyde in 15 mM phosphate buffered saline (PBS) (pH7.3) for 30 min at room temperature (RT). To enhance penetration of the cell membrane, sections were treated with 0.25% Triton-X in PBS for 10 min at RT. To reduce non-specific background staining, sections were treated with 3% goat serum and 5% bovine serum albumin (BSA) in PBS for 30 min at RT. For MAP-2 protein staining, sections were incubated with anti-MAP-2 rabbit antibody (1:200; Sigma, St. Louis, USA) in PBS containing 4% goat serum, 0.3% BSA and 0.3% Triton-X over night at 4 °C. Antibody was detected using the Vectastain ABC Elite kit (PK-6101, Vector Laboratories, CA, USA). DAB substrate kit (SK-4100, Vector Laboratories) was used for visualization.

2.3. *Real-time RT-PCR*

Quantitative real-time RT-PCR was performed in the ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) using SYBR green dye. Quantitative PCR was prepared in duplicate with 50 μl of reaction mixture in MicroAmp optical 96-well reaction plates. Each reaction well contained 12.5 μl of RNA sample, 25 μl of SYBR Green PCR Master Mix, 0.5 μl of RT Mix (Quiagen Sciences, MA, USA), 7 μl RNAase free water, and 25 pmol each of forward and reverse primers. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: [sense 5′-TGCACCACCAACTGCTTAGC-3′, antisense 5′-GGATGCAGGGATGATGTTCTG-3′], for OX1R, [sense 5′-GCATATCCACCTGGCCTGAA-3′, antisense 5′-CCACCATGCCAACGAGATCC-3′] [42], for OX2R, [sense 5′-CTACGCTCTTCTGCTATTGA-3′, antisense 5′-ACTGGCATGCTGATACATAC-3′] [41], for NR1 subunit, [sense 5′-AAGCCCAACGCCATACAGAT-3′, antisense 5′-AGGCGGGTGGCTAACTAGGA-3′] [44], for NR2A, [sense 5′-GCTACACACCCTGCACCAATT-3′, antisense 5′-CACCTGGTAACCTTCCTCAGTGA-3′] [6], for NR2B subunit, [sense 5′-CCCAACATGCTCTCTCCCTTA A-3′, antisense 5′-CAGCTAGTCGGCTCTCTTGGTT-3′] [3], for GluR1 subunit, [sense 5′-TTCCTGTTGACACATCCAATCAAT-3′, antisense 5′-ATGGTCGATAATGCTAATGAGAGCTT-3], and for GluR2 subunit, [sense 5′-CCTAGCTTCCCAACAGATGGC-3′, antisense 5′-GAGGTATGCGAACTTGTCCCA-3′]. Real-time RT-PCR was conducted according to our previous report [25]. All gene-specific mRNA expression values were normalized against the internal housekeeping gene GAPDH. The results are presented as the mean ± S.E.M. of 8–31 RNA samples per group.

2.4. *Data analysis*

Statistical analysis of the data was carried out by analysis of variance (ANOVA) followed by Dunnett's multiple range test. Statistical significance was defined as $p < 0.05$.

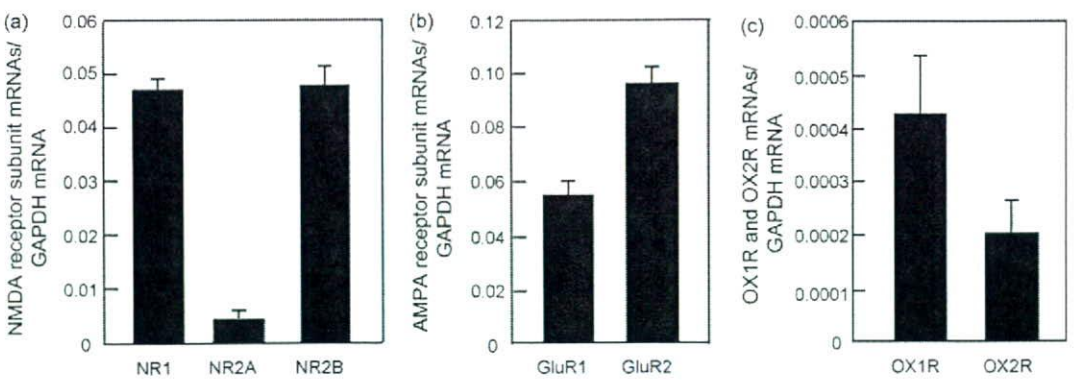

Fig. 2 – mRNA expressions of N-methyl-D-aspartate (NMDA) receptor subunits (NR1, NR2A, NR2B) (a), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor subunits (GluR1, GluR2) (b), and orexin receptors (OX1R, OX2R) (c) in rat cultured cortical neurons. Values are mean ± S.E.M. from 8 to 31 RNA samples.

3. Results

mRNAs of NMDA receptor subunits (NR1, NR2A, NR2B) and AMPA receptor subunits (GluR1, GluR2) were detected in the rat cultured primary cortical neurons (Fig. 2a and b), which agreed with previous reports [17,45]. The mRNA expressions of OX1R and OX2R were also detected in the cultured neurons (Fig. 2c).

Orexin-A at concentrations of 0.1 and 1 μM significantly decreased the mRNA expressions of NR1 ($F(6, 102) = 10.76$, $p < 0.01$; Dunnett multiple range test; 0.1 μM, $p = 0.0107$; 1 μM, $p < 0.01$) and NR2A ($F(6,114) = 15.29$, $p < 0.01$; Dunnett multiple range test; 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$) (Fig. 3a and b). Orexin-B at the concentrations 0.01, 0.1 and 1 μM significantly decreased the mRNA expressions of NR1 ($F(6,114) = 15.29$, $p < 0.01$; Dunnett multiple range test; 0.01 μM, $p = 0.0194$; 0.1 μM, $p < 0.0194$; 1 μM, $p < 0.01$) and NR2A ($F(6,114) = 15.29$, $p < 0.01$; Dunnett multiple range test; 0.01 μM, $p < 0.01$, 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$) (Fig. 3a and b). The mRNA expression of NR2B was significantly decreased by both orexin-A and orexin-B at the concentration of 1 μM ($F(6,102) = 8.22$, $p < 0.01$; Dunnett multiple range test; orexin-A, 1 μM, $p < 0.01$; orexin-B, 1 μM, $p < 0.01$) (Fig. 3c). With regard to the mRNA expressions of AMPA receptor subunits, the mRNA expressions of both GluR1 and GluR2 were significantly decreased by both orexin-A and orexin-B at 0.01, 0.1 and 1 μM (GluR1: $F(6,108) = 18.18$, $p < 0.01$; Dunnett multiple range test; orexin-A, 0.01 μM, $p < 0.01$; 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$; orexin-B, 0.01 μM, $p < 0.01$; 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$) (Fig. 3d), (GluR2: $F(6,114) = 12.92$, $p < 0.01$; Dunnett multiple range test; orexin-A, 0.01 μM, $p = 0.0215$; 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$; orexin-B, 0.01 μM, $p < 0.01$; 0.1 μM, $p < 0.01$; 1 μM, $p < 0.01$) (Fig. 3e).

While the inhibitory effect of orexin-B on the mRNA expressions of these receptor subunits was about 2-fold as potent as that of orexin-A at the same concentration, there

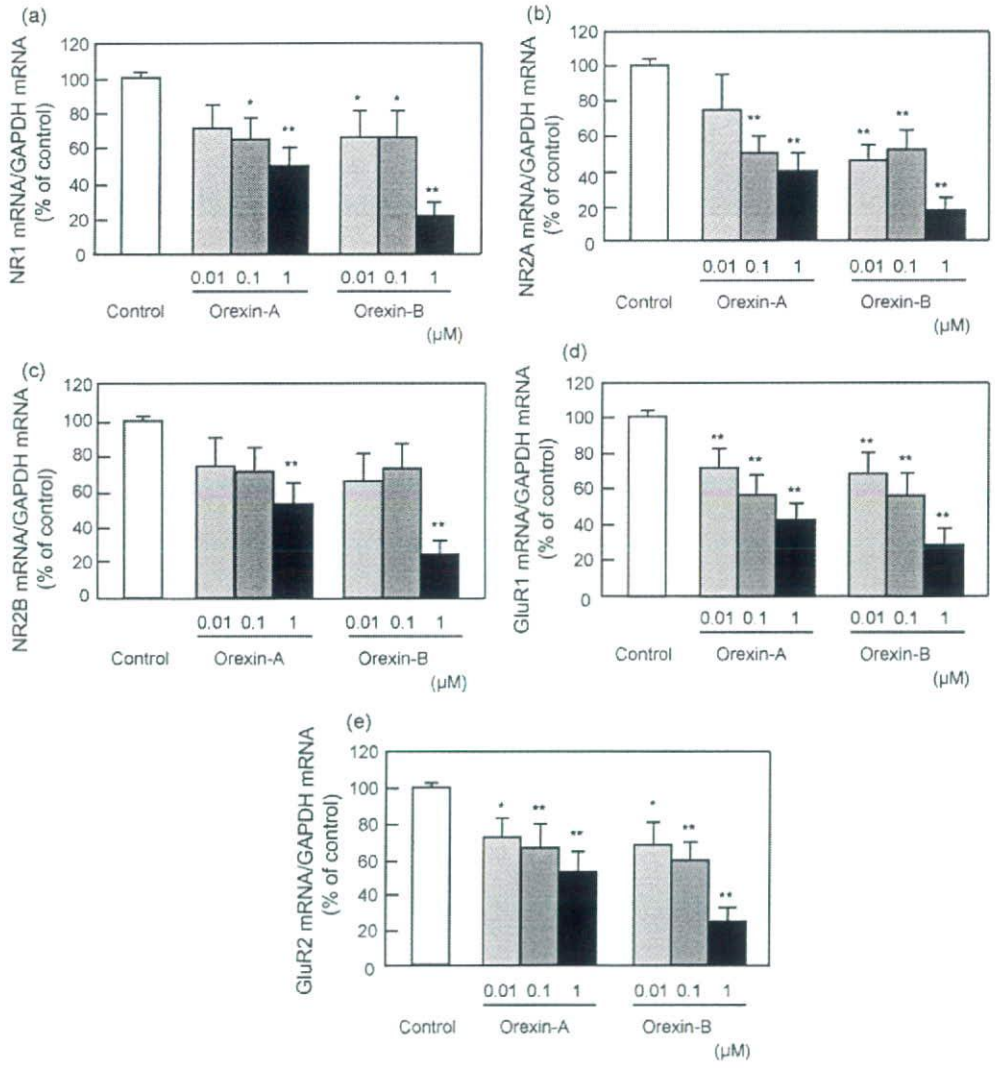

Fig. 3 – Changes of mRNA expressions of NR1 (a), NR2A (b), NR2B (c), GluR1 (d) and GluR2 (e) subunits induced by incubation for 6 days with orexin-A and orexin-B (0.01, 0.1, 1 μM). Data are presented as percent of control. The values are mean ± S.E.M. from 11 RNA samples. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group.

was no statistical difference between orexin-A and orexin-B at every concentration.

In the present experiments, neuronal degeneration was not observed at 3 days or at 6 days after the application of neuropeptides (data not shown).

4. Discussion

Recent studies have shown that central administration of orexin-A and orexin-B has effects on learning and memory. In the cerebral cortex, glutamatergic system is important in cognition. Recently it was reported that the feeding response to orexin-A in the perifornical region of the lateral hypothalamus is mediated by the glutamatergic systems, especially NMDA receptors [14]. Several lines of increasing evidence indicate that orexin modulates plasticity and NMDA receptor currents in the hippocampus [2,37] and potentiates glutamatergic transmission in prefrontal cortex [27]. In this study, orexins were applied to rat cultured primary cortical neurons for 6 days to evaluate their chronic effect because cortical neurons of obese animals are supposed to be chronically stimulated by the high concentrations of orexins up-regulated in the brain of obese animals. The present study demonstrated that orexin-A and orexin-B significantly down-regulated the mRNA expressions of NMDA and AMPA receptor subunits in rat cultured primary cortical neurons on 6-day application. These findings indicate that orexin might modulate the excitatory transmission in the cerebral cortex by altering the subunit ratio of NMDA and AMPA receptors.

Several lines of evidence indicate that the ability of the NR2A subunit to modulate NMDA receptor currents may be involved in the modulation of thresholds for long-term potentiation (LTP) and learning behavior [23,43]. Moreover, the NR2B transgenic mice, in which the NMDA receptor function is enhanced by the NR2B subunit transgene in neurons of the forebrain, were reported to show enhancement of cognition [39]. These findings show that NR2A and NR2B subunits play a pivotal role in learning and memory processing. In this regard, orexin has been reported to increase plasticity and NMDA receptor currents in the hippocampus and potentiates glutamatergic transmission in the prefrontal cortex in electrophysiological studies [2,27,37], suggesting that orexin may enhance cognitive abilities. Although recent behavioral studies have shown that central administration of orexin-A and orexin-B have effects on learning and memory, the literature concerning the role of the orexin system in cognition remains controversial. Aou et al. have reported that intracerebroventricular administration of orexin-A produced memory impairment in water maze performance in a 2-day training protocol [2], which may be supported by the present findings demonstrating the down-regulation of NMDA receptor subunits, NR1, NR2A and NR2B by orexin. In contrast, Akbari et al. reported that blockade of OX1R in the CA1 region or dentate gyrus of the hippocampus impaired memory processing in the Morris Water Maze task [1]. The discrepancy may be attributed to the different action in each region or different experimental methods.

The subunit of the AMPA receptor, GluR2 was reported to be involved in regulation of synaptic plasticity and sustaining LTP [11,43]. On the other hand, the blockage of AMPA receptor by the antagonists, CNQX and NBQX in rats were demonstrated to induce cognitive impairment [6,36]. Therefore, the AMPA receptor is also demonstrated to be potentially involved in learning and memory processes. It was recently demonstrated that orexin neurons robustly express neuronal activity-regulated pentraxin (Narp), secreted neuronal pentraxin, which has been implicated in regulating clustering of AMPA receptors [32]. The present findings with rat neuron cultures, showing that orexins exhibited down-regulation of mRNA expressions of the AMPA receptor subunits GluR1 and GluR2, suggest the possibility of impairment of AMPA receptor functions.

Orexin, one of the orexigenic neuropeptides in the hypothalamus, projects neurons from the lateral hypothalamus to the cerebral cortex and hippocampus. These observations including our present results indicate that orexin may have several regulatory actions on NMDA receptor and AMPA receptor activities. Further detailed experiments are needed to elucidate the pathophysiological significance of orexins in the hypothalamo-cortical system.

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Orexins increase mRNA expressions of neurotrophin-3 in rat primary cortical neuron cultures

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ABSTRACT

Orexins and melanin-concentrating hormone (MCH) as orexigenic neuropeptides are present in the lateral hypothalamus, and their receptors are distributed in the cerebral cortex and hippocampus. In the present study, the regulatory effects of orexin-A, orexin-B and MCH on neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) expressions were examined in primary cortical neuron cultures using quantitative real-time PCR. Both orexin-A and orexin-B on 6-day exposure significantly increased the NT-3 mRNA at concentrations of 0.01, 0.1 and 1 μM. Orexin-A and B at 1 μM led to an increase of twofold or more over the control. However, no such NT-s mRNA increase occurred with exposure to MCH at the same concentrations as orexins. The mRNA expression of BDNF was significantly increased only by orexin-B at 1 μM. These findings suggest that orexins, but not MCH, may be an inducer of NT-3 in the cerebral cortex.

Orexins (orexin-A, orexin-B) and melanin-concentrating hormone (MCH) are orexigenic neuropeptides predominantly present in the lateral hypothalamus [23]. The orexin receptors (OX1R, OX2R) and MCH receptors (MCHR1) are distributed at high concentrations in the cerebral cortex and hippocampus [12]. These areas are postulated to play an important role in regulating the higher functions of the central nervous system, such as learning and memory, based on neuronal plasticity. In this regard, orexin has been reported to play a critical role in neuronal plasticity relevant to addiction in the ventral tegmental area and in long-term potentiation of synaptic transmission in the hippocampus [4,24]. MCH was also found to increase hippocampal synaptic transmission via increased synaptic efficacy [29]. Recently, we demonstrated that in rat primary cortical neuron cultures orexins and MCH decreased the expression of subunits of the NMDA (*N*-methyl-d-aspartate) receptor and the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor [31]. These findings suggest that orexin and MCH may regulate the higher functions, such as cognition and emotion, of the central nervous system as well as energy regulation. Moreover, neurotrophic factors also play an important role in regulating neuronal plasticity in the brain. The neurotrophin family includes brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) and neurotrophin-5 (NT-5) [15]. They exert their biological functions via each of the specific tyrosine kinase receptors (Trk) [2,5]. In the mature nervous system, neurotrophic factors, especially BDNF and NT-3, are demonstrated to be widely distributed in the brain, where they regulate the activity-dependent synaptic plasticity which is involved in the learning and memory regulation [22,27].

In this study, to explore the functions of orexin and MCH in the cerebral cortex, we examined the effects of chronic application of orexin and MCH on NT-3 and BDNF mRNA expressions using primary cortical neuron cultures.

For preparing rat cultured primary cortical neurons timed pregnant Sprague–Dawley rats were obtained from Japan SLC, Inc. (Japan) on gestational day 18. The animals were anesthetized with pentobarbital sodium (50 mg/kg, ip; Abbott, Abbott Park, IL, USA) and sacrificed by cervical dislocation. The fetuses were delivered and decapitated. In each experiment, fetuses were extracted from four maternal rats. All experiments were performed in accordance with the guideline established by the Institutional Animal Investigation Committee at Kyoto University (Med Kyo 06514), Chiba

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University (2006362). Every effort was made to optimize the comfort and to minimize the use of animals. According to our previous report [31], the cultured primary cortical neurons were prepared. Briefly, the cerebral cortex was dissected and cut into small pieces in Hanks' balanced salt solution (HBSS: Ca^{2+} and Mg^{2+} free) (Invitrogen, Carlsbad, CA, USA). The tissue was then dispersed with 0.025% trypsin-EDTA solutions (Invitrogen, Carlsbad, CA USA). The cell suspension was centrifuged at 800 × *g* for 5 min. The cells were suspended in Neurobasal Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2% of B27 containing antioxidants (Invitrogen, Carlsbad, CA, USA), L-glutamine (final concentration 0.5 mM) and antibiotic–antimycotic solution (final concentration 1%) (Nacalai tesque, Kyoto, Japan). The cell suspension was centrifuged at 800 × *g* for 5 min. Live cells were counted using a hemocytometer, and the cell suspension was then diluted with Neurobasal Medium at 2×10^6 cells/ml. Cells were seeded onto poly-D-lysine-coated 6-well plates (BD Bioscience, Discovery Labware, Bedford, MA, USA) in 2×10^6 cells/ml well. All cultures were maintained in Neurobasal Medium at 37 °C in 95% humidified air and 5% CO_2. At the 3rd day of culture, cytosine β-D-arabino-furanoside hydrochloride (final concentration 10 μM) (Sigma, St. Louis, MO, USA), a selective inhibitor of DNA synthesis, was added for 72 h in culture to prevent further proliferation of non-neuronal cells.

Orexin-A, orexin-B and MCH (0.01, 0.1 and 1 μM) were applied to cultured wells on the 6th day of culture, and the cultured cells were incubated for 6 days. Cultured medium which contained neuropeptides was changed every 3 days. On the 12th day of culture, the total cellular RNA was extracted from two cultured wells as one RNA sample using an RNeasy Mini Kit (Quiagen Sciences, MA, USA). The samples were stored at −20 °C until assay. All experiments consisted of three to six repetitive runs. For each experiment, two to six mRNA were obtained. Orexin-A, orexin-B and MCH were purchased from Peptide Institute Inc. (Osaka, Japan). Neuronal degeneration was assessed every 3 days after neuropeptide application using the efflux of lactate dehydrogenase by CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Co., Madison, WI, USA) [31].

Quantitative real-time RT-PCR was conducted according to our previous report [31]. Quantitative real-time RT-PCR was performed with the ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) using SYBR green dye. Quantitative PCR was conducted in duplicate with 50 μl of reaction mixture in MicroAmp optical 96-well reaction plates. Each reaction well contained 12.5 μl of RNA sample, 25 μl of SYBR Green PCR Master Mix, 0.5 μl of RT Mix (Quiagen Sciences, MA, USA), 7 μl RNAase free water, and 25 pmol each of forward and reverse primers. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: [sense 5′-TGCACCACCAACTGCTTAGC-3′, antisense 5′-GGATGCAGGGATGATGTTCTG-3′], for OX1R, [sense 5′-GCATATCCACCTGGCCTGAA-3′, antisense 5′-CCACCATGCCAACGAGATCC-3′] [28], for OX2R, [sense 5′-CTACGCTCTTCTGCTATTGA-3′, antisense 5′-ACTGGCATGCTGATACATAC-3′] [28], for MCHR1, [sense 5′-TCA GCT TGG GCT ATG CTA ACA G-3′, antisense 5′-CAA CAC CAA GCG TTT TCG AA-3′ [8], for BDNF, [sense 5′-GGTCACAGTCCTGGAGAAAG-3′, antisense 5′-GCTTATCCTTATGAACCGCC-3] [30], and for NT-3, [sense 5′-TGCAGAGCATAAGAGTCACC-3′, antisense 5′-AAGTCAGTGCTCGGACGTAG-3′] [30]. All gene-specific mRNA expression values were normalized against the internal housekeeping gene GAPDH.

The results are presented as the mean ± S.E.M. of 8–31 RNA samples per group. Statistical analysis of the data was carried out by analysis of variance (ANOVA) followed by Dunnett's multiple range test. Statistical significance was defined as $p < 0.05$.

Quantitative RT-PCR analysis showed the presence of mRNAs of orexin receptors (OXR1, OXR2) and MCH receptor (MCHR1) in rat primary cortical neuron cultures used in this study (Fig. 1). Moreover, the basal expressions of NT-3 and BDNF mRNAs were also detected in the cultured cortical neurons (Fig. 2).

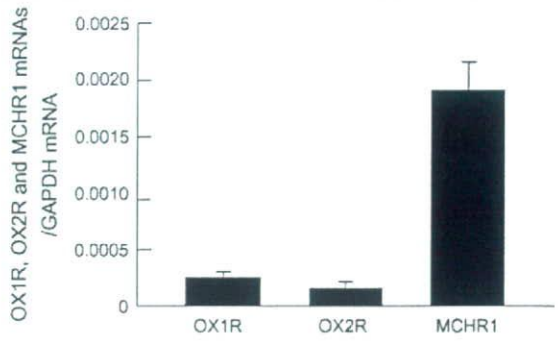

Fig. 1. mRNA expressions of orexin receptors (OX1R, OX2R) and MCHR1 in rat cultured cortical neurons. The values are mean ± S.E.M. from 9 to 10 RNA samples.

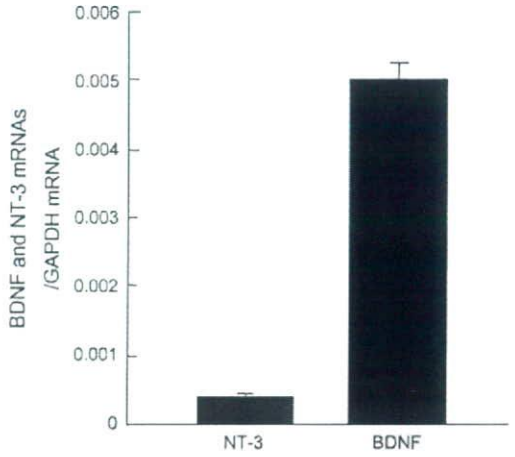

Fig. 2. mRNA expressions of BDNF and NT-3 in rat cultured cortical neurons. The values are mean ± S.E.M. from 8 to 31 RNA samples.

The mRNA expression of NT-3 following 6-day exposure to orexin-A at 0.01, 0.1 and 1 μM markedly increased to 1.57, 1.84 and 2.31 of control levels, respectively (Fig. 3). In a similar manner, orexin-B at 0.01, 0.1 and 1 μM also led to significant increases to 1.55, 1.63 and 2.49-fold of the control levels, respectively (Fig. 3).

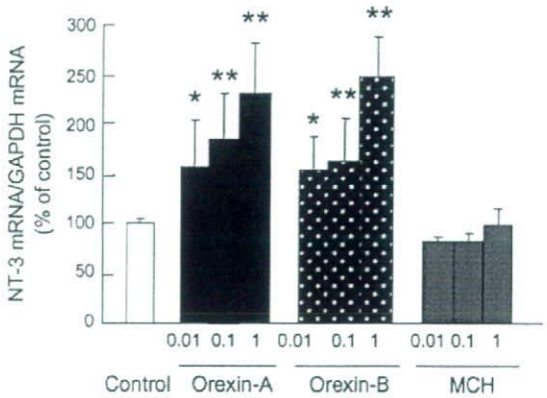

Fig. 3. Changes of mRNA expression of NT-3 induced by incubation for 6 days with orexin-A, orexin-B and MCH (0.01, 0.1, and 1 μM). Data are presented as a percentage of the control. The values are mean ± S.E.M. from 11 RNA samples. $^*p < 0.05$, $^{**}p < 0.01$ vs. control group.

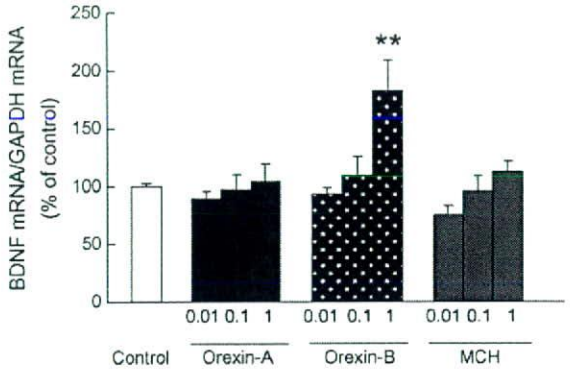

Fig. 4. Changes of mRNA expression of BDNF induced by incubation for 6 days with orexin-A, orexin-B and MCH (0.01, 0.1, and 1 μM). Data are presented as a percentage of the control. The values are mean ± S.E.M. from 11 RNA samples. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group.

On the other hand, no change in the mRNA expression of NT-3 occurred on exposure to MCH (Fig. 3). The effects of these neuropeptides on BDNF mRNA expression were examined with the same RNA samples. Orexin-B at 1 μM significantly increased BDNF mRNA to 1.82-fold the control level, but its lower concentrations did not affect the mRNA expression of BDNF (Fig. 4). Neither orexin-A nor MCH changed the BDNF mRNA expression (Fig. 4).

In the present study, neuronal degeneration was not detected at 3 or 6 days after the application of neuropeptides (data not shown).

In the present study, the mRNA expression of NT-3 in primary cortical neuron cultures was markedly increased at 6 days after application of orexin-A and B, but not MCH. Moreover, only a high concentration of orexin-B significantly increased the expression of BDNF mRNA, while orexin-A and MCH did not. These findings suggest that orexins may be potent inducers of NT-3 in the cerebral cortex. Orexin and MCH are restrictedly present in the lateral hypothalamus and their neurons project to the cerebral cortex and hippocampus which contain their receptors. The radioimmunoreactive contents of orexins and MCH in the lateral hypothalamus have been shown to significantly increase in obese rodent models [19].

In the adult brain, especially in the cerebral cortex and hippocampus, BDNF and NT-3 act as neurotransmitter and neuromodulator in the central nervous system, and they act on TrkB and TrkC [5,25], respectively. Both BDNF and NT-3 have been implicated in the genesis of new synapses, which may be important for structural aspects of neuronal plasticity [18]. Neurotrophin expression in neurons is mainly regulated by neuronal depolarization [10,13], which may be an important mechanism in neuronal plasticity and may influence neuronal susceptibility to excitotoxicity. Chronic depolarization induced by K^+ (25 mM) in primary cultures of rat cerebellar neurons sustained a persistent increase of BDNF expression which is accompanied by a drastic decrease in NT-3 expression [7,10]. In contrast to the up-regulation of BDNF mRNA, the level of NT-3 mRNA does not change either after injection of kainic acid [1] or after kindled seizures [9]. Moreover, Rocamora et al. [21] have shown with an experimental model of limbic seizures that the dramatic increase of NGF and BDNF expression is accompanied by a fivefold decrease of NT-3 mRNA in dentate gyrus granule cells [21]. The reciprocal regulation of BDNF and NT-3 has also been observed in the dentate gyrus granule cells following cerebral ischemia [16,26]. Reduction of NT-3 mRNA in the hippocampal dentate gyrus was also demonstrated after long-term potentiation [6] and status epileptitus [3,20].

Several lines of evidence demonstrate the involvement of the glutamate nervous system in BDNF expression. Activation of the NMDA receptor, an ionotrophic glutamate receptor, increases BDNF gene expression in cortical neuron cultures [11]. Continuous culture exposure to non-toxic concentrations of NMDA resulted in a prolonged increase in BDNF mRNA expression in primary cultures of rat cerebellar granule neurons. In addition, AMPA also induced a concentration-dependent increase in BDNF mRNA and protein expression [17]. Moreover, an AMPA receptor potentiator (LY392098) was reported to increase BDNF mRNA levels, while it did not change in either NT-3 or NT-4 mRNA. Activation of both L-type Ca^{2+} channels and mitogen-activated protein (MAP) kinases contribute to AMPA receptor-mediated increases in BDNF mRNA [14]. AMPA antagonist CNQX, but not MK-801, suppresses kinase-induced increases in BDNF mRNA in hippocampal neuron cultures [32], while the activation of GABAergic transmission reduces the mRNA levels of BDNF [33]. These findings indicate that the neural depolarization induced by the activation of glutamate receptors increases BDNF expression.

These above-mentioned observations clearly demonstrate that NT-3 expression is suppressed by neuronal depolarization and, moreover, reciprocally regulated in contrast to BDNF expression in the brain. However, in the present experiment using primary cultured cortical neurons, orexin-A and B significantly up-regulated NT-3 mRNA expression, and orexin-B, to a lesser but significant extent, up-regulated the expression of BDNF mRNA. Although the mechanisms leading to reduced NT-3 mRNA expression induced by orexins has not yet been elucidated, orexins may be potent inducers of NT-3 in the cerebral cortex.

As above mentioned NT-3 as well as BDNF plays an important role in neurotransmission and neuronal plasticity in the brain, and its synthesis and release are regulated by neuronal depolarization. The present study demonstrated that orexins, but not MCH, increased the expression of NT-3 mRNA. It seems likely orexin might act on some neurons which was different from neurons containing MCH receptors. Orexin is well known as orexigenic neuropeptide in the hypothalamus and is regulated by hunger status. Orexin released in response to the peripheral metabolic signals may increase NT-3 mRNA in the cerebral cortex, resulting in the modulation of neuronal transmission in the cerebral cortex.

The findings in the present study indicate that these neuropeptides involved in energy regulation may regulate the expressions of NT-3 and BDNF in the cerebral cortex, indicating that these neuropeptides can regulate the activity of the cerebral cortex via changes in neuronal plasticity. These findings offer information for understanding the functional significance of NT-3 in obese animals and a new insight into the bidirectional interaction between energy regulation and higher functions of the limbic system, such as learning/memory and emotion.

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ARTICLE

Beneficial effects of leptin on glycaemic and lipid control in a mouse model of type 2 diabetes with increased adiposity induced by streptozotocin and a high-fat diet

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Abstract
Aims/hypothesis We have previously demonstrated the therapeutic usefulness of leptin in lipoatrophic diabetes and insulin-deficient diabetes in mouse models and could also demonstrate its dramatic effects on lipoatrophic diabetes in humans. The aim of the present study was to explore the therapeutic usefulness of leptin in a mouse model of type 2 diabetes with increased adiposity.
Methods To generate a mouse model mimicking human type 2 diabetes with increased adiposity, we used a combination of low-dose streptozotocin (STZ, 120 μg/g body weight) and high-fat diet (HFD, 45% of energy as fat). Recombinant mouse leptin was infused chronically (20 ng [g body weight]$^{-1}$ h^{-1}) for 14 days using a mini-osmotic pump. The effects of leptin on food intake, body weight, metabolic variables, tissue triacylglycerol content and AMP-activated protein kinase (AMPK) activity were examined.
Results Low-dose STZ injection led to a substantial reduction of plasma insulin levels and hyperglycaemia. Subsequent HFD feeding increased adiposity and induced insulin resistance and further augmentation of hyperglycaemia. In this model mouse mimicking human type 2 diabetes (STZ/HFD), continuous leptin infusion reduced food intake and body weight and improved glucose and lipid metabolism with enhancement of insulin sensitivity. Leptin also decreased liver and skeletal muscle triacylglycerol content accompanied by an increase of α2 AMPK activity in skeletal muscle. Pair-feeding experiments demonstrated that leptin improved glucose and lipid metabolism independently of the food intake reduction.
Conclusions/interpretation This study demonstrates the beneficial effects of leptin on glycaemic and lipid control in a mouse model of type 2 diabetes with increased adiposity, indicating the possible clinical usefulness of leptin as a new glucose-lowering drug in humans.

Keywords High-fat diet · Insulin sensitivity · Leptin · Overweight · Streptozotocin · Tissue triacylglycerol content · Type 2 diabetes

Abbreviations

AMPK	AMP-activated protein kinase
GTT	glucose tolerance test
HFD	high-fat diet
SD	standard diet
STZ	streptozotocin

Introduction

Leptin is an adipocyte-derived hormone that plays a key role in regulating food intake and energy expenditure, and participates in increasing glucose metabolism [1, 2]. Leptin deficiency causes obesity, insulin resistance and diabetes in mice and humans [3–5]. We previously generated transgenic skinny mice (LepTg) overexpressing leptin under the control of the liver-specific human serum amyloid P component promoter [6]. LepTg mice showed elevated

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plasma leptin levels comparable to those of obese human individuals, providing a unique experimental model to investigate various actions of leptin [6–11]. LepTg mice exhibited increased glucose metabolism and insulin sensitivity with augmented liver and skeletal muscle insulin receptor signalling [6]. LepTg mice also exhibited increased lipid metabolism accompanied by increased lipoprotein lipase activity and clearance of triacylglycerol [7]. In addition, LepTg mice had reduced tissue triacylglycerol content along with increased energy expenditure through augmented phosphorylation of AMP-activated protein kinase (AMPK), a key enzyme that mediates the leptin effect on fatty acid β-oxidation in skeletal muscle [8, 9]. Therefore, these findings led us to hypothesise that leptin acts as a glucose-lowering drug with a lipid-lowering effect in vivo.

Given the glucose-lowering action of leptin, we and others have demonstrated that leptin infusion or transgenic overexpression of leptin reverses metabolic abnormalities in different mouse models of lipodystrophy [10, 12]. Recently, we and others confirmed that leptin treatment effectively reduces food intake and improves hyperglycaemia, hypertriacylglycerolaemia and fatty liver in patients with lipoatrophic diabetes [13–16]. In addition, we demonstrated that leptin is useful as a glucose-lowering agent in a mouse model of insulin-deficient diabetes induced by high-dose streptozotocin (STZ) [11]. Leptin infusion reduced the dose of insulin required to improve hyperglycaemia by more than 90%, and prevented insulin-induced body weight gain in STZ-injected mice. However, the therapeutic usefulness of leptin in type 2 diabetes, a more prevalent form of diabetes, remains unclear.

In patients with type 2 diabetes, impaired insulin secretion caused by beta cell dysfunction and insulin resistance in target tissues contributes to increased blood glucose levels [17]. Patients with type 2 diabetes often exhibit dyslipidaemia and an increase of triacylglycerol content in the liver and skeletal muscle [18, 19]. Furthermore, in contrast to patients with lipoatrophic diabetes and insulin-deficient diabetes who are in hypoleptinaemic states [13–16, 20], patients with type 2 diabetes often have increased adiposity and elevated leptin levels.

Previous studies have shown that low-dose STZ injection leads to the partial destruction of pancreatic beta cells and a high-fat diet (HFD) induces insulin resistance in rodents [21–23]. The degree of beta cell destruction and insulin resistance can be adjusted by dosage, duration and condition of STZ injection and HFD feeding [11, 24]. The effects of various glucose-lowering drugs (sulfonylurea, metformin, thiazolidinedione etc) have been examined in mice treated with low-dose STZ and HFD as a model of type 2 diabetes [22, 23]. In the present study, we too generated a mouse model mimicking human type 2 diabetes using low-dose STZ and HFD to examine the effect of leptin infusion. STZ/HFD mice exhibited increased adiposity and disorders in glucose and lipid metabolism accompanied by impaired insulin secretion and insulin resistance. We report here the beneficial effects of leptin infusion on glycaemic and lipid control in this mouse model of type 2 diabetes with increased adiposity.

Methods

Animals Seven-week-old male C57BL/6J mice were purchased from Japan SLC, Shizuoka, Japan. The mice were caged individually and kept under a 12 h light–dark cycle (light on at 09:00 hours) with free access to water and standard diet (SD) (NMF, 14.6 kJ/g, 13% of energy as fat; Oriental Yeast Co., Tokyo, Japan) unless otherwise stated. Animal care and all experiments were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Generation of a mouse model of type 2 diabetes One week after purchase, mice were injected i.p. once with vehicle or low-dose STZ (120 μg/g body weight in 10 mmol/l sodium citrate buffer, pH 4.0; Sigma-Aldrich, St Louis, MO, USA) after 4 h of fasting. After 3 weeks, the vehicle-injected mice were randomly divided and placed on SD or HFD (D12451, 19.7 kJ/g, 45% of energy as fat; Research Diets, New Brunswick, NJ, USA) (termed control and HFD mice, respectively), and the STZ-injected mice with similar degrees of hyperglycaemia and body weight were also randomly divided and placed on SD or HFD (termed STZ and STZ/HFD mice, respectively). Each group of mice was fed with either diet for 5 weeks before they were used for the leptin infusion experiment.

Leptin infusion experiments On day 0, a mini-osmotic pump (Alzet model 2002; Alza, Palo Alto, CA, USA) was implanted s.c. in the mid-scapular region of each mouse. The pump delivered saline or recombinant mouse leptin (Amgen, Thousand Oaks, CA, USA) (20 ng [g body weight]$^{-1}$ h^{-1}) s.c. for 14 days. SD or HFD feeding was continued during the leptin infusion experiment.

Food intake, body weight and per cent body fat Food intake was measured before and during the leptin infusion experiment. Body weight was measured on days 0 and 14. Per cent body fat was measured before the leptin infusion experiment under pentobarbital anaesthesia (Nembutal; Dainippon Sumitomo Pharma, Osaka, Japan), using a Latheta LTC-100 (Aloka, Tokyo, Japan).