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Changes in circulating cytokine levels in midlife women with psychological symptoms with selective serotonin reuptake inhibitor and Japanese traditional medicine

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ABSTRACT

Objective: The aim of the present study was to compare the effects on serum cytokine concentrations of paroxetine, a selective serotonin re-uptake inhibitor, and kamishoyosan, a Japanese traditional medicine, in midlife women with psychological symptoms.
Methods: Seventy-six women with psychological symptoms such as anxiety and mild depression as menopausal symptoms were enrolled in this study. Thirty-eight women received oral administration of 10 mg paroxetine every day, and 38 women received oral administration of kamshoyosan every day for 6 months. Overall climacteric symptoms were assessed using Greene's climacteric scale. Serum levels of cytokines were measured using a multiplexed human cytokine assay.
Results: Greene's total scores in both women treated with paroxetine and in women treated with kamishoyosan decreased significantly. Percentage decreases in Greene's total, psychological and vasomotor scores during the 6-month period in the paroxetine group were significantly greater than those in the kamishoyosan group. Serum IL-6 concentration in women treated with paroxetine decreased significantly. Serum concentrations of IL-8, IL-10, macrophage inflammatory protein (MIP)-1β and monocyte chemoattractant protein-1 in women treated with paroxetine decreased significantly. On the other hand, serum IL-6 concentration in women treated with kamishoyosan decreased significantly, but other serum concentrations did not change significantly.
Conclusion: Decrease in IL-6 concentration may be involved in the mechanism of the actions of both paroxetine and kamishoyosan in women with psychological symptoms, and IL-6 may therefore be useful as a marker of treatment. The action of paroxetine may also be associated with decreases in IL-8, IL-10, MIP-1β.

1. Introduction

In midlife women during the menopausal transition, psychological symptoms such as anxiety and mild depression as well as vasomotor symptoms have been observed as menopausal symptoms. A selective serotonin reuptake inhibitor (SSRI) has been used to treat depression in women, but adverse reactions such as nausea and headache have been observed in women treated with SSRI [1].

In Japan, various Japanese traditional medicines have been used for treating women who complain of menopausal symptoms. Kamishoyosan (Jia-wei-xiao-yao-san) is one of the formulae used for treatment of psychological symptoms such as anxiety, depression and irritability in menopausal women [2,3]. Recently, it has been reported that women with premenstrual dysphoric disorder were successfully treated with kamishoyosan [4]. Kamishoyosan consists of the following 10 medical herbs: Bupleurum root, Peony root, Atractylodis lanceae rhizome, Angelica root, Hoelen, Gardenia fruit, Moutan bark, Glycyrrhiza root, Ginger rhizome and Mentha herb. It is thought that kamishoyosan acts on the central nervous system, but the mechanism of the action of kamishoyosan has not been fully elucidated.

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Cytokines are involved in various functions of the central nervous system. It has been reported that circulating cytokines are dysregulated in major depression [5]. Plasma interleukin (IL)-6 concentration has been reported to be increased in major depressive disorders [6,7]. Levels of mitogen-induced cytokines such as IL-1β, IL-2, IL-10 and interferon (IFN)-γ have also been reported to be high in patients with major depression [8]. In midlife women with depression as a menopausal symptom, plasma IL-6 concentration was found to be increased [9]. We also reported that serum concentrations of IL-6, IL-8 and IL-10 were high in midlife women with psychological symptoms [10]. On the other hand, it has been reported that decreases in serum concentrations of IL-6 and tumor necrosis factor (TNF)-α were observed in depressed patients treated with SSRI [11,12]. Ushiroyama et al. reported that plasma TNF-α concentration was increased in depressed menopausal women treated with kamishoyosan [3]. However, the changes in cytokines in women treated with paroxetine and kamishoyosan have not been fully elucidated.

To date, it has been difficult to detect low levels of circulating cytokines in serum of healthy women. Recently, a multiplexed cytokine assay for measurement of serum concentrations of cytokines has been developed, and the use of this assay has enabled simultaneous measurements of low levels of various cytokines in serum of healthy subjects [13,14].

In the present study, we compared the effects of paroxetine and kamishoyosan on serum cytokine concentrations in midlife women with psychological symptoms using a highly sensitive multiplexed cytokine assay.

2. Subjects and methods

2.1. Subjects

The subjects of this study were recruited from patients visiting the outpatient clinic of the Department of Obstetrics and Gynecology, Tokushima University Hospital. Seventy-six women who had complained of psychological symptoms such as anxiety and mild depression as menopausal symptoms were enrolled in this study between November 2005 and October 2007. Informed consent for participation in this study was obtained from each woman. The Ethics Committee of Tokushima University Hospital approved the study. Women with major depression were excluded. Reviews of medical histories and the results of physical examinations and blood chemistry tests showed that all of the women were in good health. None of the subjects had taken any medication known to influence the immune system for at least 1 year. Subjects suspected of having infectious diseases, inflammatory disorders, malignancy or autoimmune diseases, of being undernourished, or of abusing alcohol or drugs were excluded according to the SENIEUR protocol [15]. Seven premenopausal women had regular menstruation and 32 perimenopausal women had experienced alterations in menstrual frequency and/or flow in the 12 months preceding entry into the study, and natural menopause had occurred in 37 women at least 12 months before entry into the study. Eligible women were randomly assigned in open, parallel-group fashion to a paroxetine group or kamishoyosan group. Thirty-eight women received oral administration of 10 mg paroxetine (Glaxo) every day and 38 women received oral administration of 7.5 g kamishoyosan (Tsumura Co., Tokyo, Japan) every day for 6 months. Climacteric symptoms were assessed using Greene's climacteric scale [16]. Compliance was assessed by pill count or sheet count, and side effects were ascertained by questionnaires at 4-week intervals. Venous blood samples were drawn into tubes between 8 a.m. and 10 a.m. after a 12-h fasting before and at 6 months of treatment. Samples obtained were frozen at −70 °C until use for analysis.

2.2. Preparation of herbal drugs

Kamishoyosan is composed of 10 medical herbs: 3 g of Bupleurum root, Peony root, Atractylodis lanceae rhizome, Japanese Angelica root, and Hoelen; 2 g of Gardenia fruit and Moutan bark; 1.5 g of Glycyrrhiza root and 1 g of Ginger rhizome and Mentha herb. Kamishoyosan used in the present study was prepared as a spray-dried powder from hot water extract and obtained from Tsumura Co. Ltd. (Tokyo, Japan).

2.3. Measurement of serum cytokine concentrations

Serum concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, TNF-α, IFN-γ, macrophage inflammatory protein (MIP)-1β and monocyte chemotactic protein (MCP)-1 were measured by using a Bio-Plex human multi-plex cytokine assay kit (Bio-Rad Laboratories, Hercules, CA, USA) as previously reported [14]. The intra- and inter-assay coefficients of variation were 2.0–7.0% and 5.2–16.1%, respectively. The sensitivity levels were 1.1 pg/ml for IL-2, IL-6 and MIP-1β, 0.5 pg/ml for IL-4, IL-7 and IL-8, 0.8 pg/ml for IL-1β and IL-5, 0.9 pg/ml for IL-10, 19.3 pg/ml for IFN-γ, 3.0 pg/ml for TNF-α and 6.7 pg/ml for MCP-1.

2.4. Measurements of concentrations of estradiol and FSH

Serum estradiol concentration was measured by a two-site immunoenzymometric assay using a commercially available kit (TOSOH Co., Tokyo, Japan). The intra- and inter-assay coefficients of variation were 4–9% and 6–9%, respectively, and the detection limit was 20 pg/ml. Serum FSH concentration was measured by an immunoradiometric assay using a commercially available kit (TFB Co., Tokyo, Japan). The intra- and inter-assay coefficients of variation were 3–4% and 3–4%, respectively, and the detection limit was 1.0 IU/l.

2.5. Analysis of kamishoyosan by HPLC

Kamishoyosan was extracted with 20 ml of methanol under ultrasonication for 30 min. The solution was filtered and subjected to treatment with an alumina cartridge (Bond Elute Co. Ltd.). Elution provided the alkaloid fraction. The methanol solution and the alkaloid fraction were tested. HPLC with an LC-10AD pump (Shimazu, Tokyo, Japan) and SPD-M10A absorbance detector was performed using a TSK-GEL ODS-80TM column (150 mm × 4.6 mm). The effluent from the column was monitored at 254 nm with a UV detector.

2.6. Statistical analysis

Based on results of the previous study [17], sample size was estimated to detect at least 20% change in levels of cytokines and chemokines after administration with 80% power at the 0.05 level of significance. We defined the values below the detection limit as half of the detection limit in further analyses. Differences between the paroxetine group and the kamishoyosan group in subject's characteristics, baseline serum hormonal concentrations and Greene's scores and percentage changes in Greene's scores were analyzed by an unpaired *t*-test, and values are presented as means ± standard deviations. Baseline serum cytokine levels, which were not normally distributed, are presented as medians with 10th and 90th percentile ranges, and significance of those values was evaluated by the non-parametric Wilcoxon rank sum test. Changes by treatments in Greene's scores were analyzed by Student's paired *t*-test, and changes by treatments in serum cytokine levels were analyzed by the non-parametric Wilcoxon signed-rank test. The relationship among continuous variables was determined by using Spearman's rank order analysis. *p* values less than 0.05 were considered to be

Table 1
Baseline characteristics in women treated with paroxetine and kamishoyosan.

	Paroxetine	Kamishoyosan	*p* values
Number	38	38	
Age (years)	50.5 (5.4)	51.4 (5.1)	0.42
Menopausal status			
Premenopause	4	3	
Perimenopause	16	16	
Postmenopause	18	19	
BMI	21.9 (3.4)	21.8 (6.7)	0.92
FSH (mIU/ml)	58.2 (40.5)	70.4 (43.1)	0.21
Estradiol (pg/ml)	55.9 (49.9)	40.2 (37.9)	0.18

Values in age, BMI, FSH and estradiol are means (standard deviations). Values in menopausal status are numbers. BMI: body mass index, FSH: follicle-stimulating hormone.

statistically significant. Box plots show median, 25th and 75th percentiles as boxes and 10th and 90th percentiles as error bars.

3. Results

3.1. General characteristics

As shown in Table 1, there were no significant differences between baseline characteristics such as age, BMI, serum concentrations of FSH and estradiol in the two groups. The proportions of pre-, peri- and postmenopausal women treated with paroxetine were 10.5%, 42.1% and 47.3%, respectively, and the proportions of pre-, peri- and postmenopausal women treated with kamishoyosan were 7.9%, 42.1% and 50.0%, respectively. Sixty-seven of the 76 women completed the 6-month study. Six of the 38 women treated with paroxetine dropped out of the study because of the following adverse effects: headache, nausea and abnormal feeling of gastrointestinal tract. One woman treated with kamishoyosan dropped out of the study because of oral bitterness and diarrhea, and two women dropped out because of no response to kamishoyosan. Data from 67 of the 76 women were therefore used for analysis.

3.2. Changes in Greene's scores in women treated with paroxetine and kamishoyosan

There were no significant differences in total Greene's scores in women before treatments with paroxetine and kamishoyosan. The mean psychological, somatic and vasomotor scores in women before treatments were also not significantly different in the two groups. As shown in Table 2, Greene's total score (mean ± standard deviation) in women treated with paroxetine was significantly

Table 2
Greene's scores before and at 6 months after treatments with paroxetine and kamishoyosan in women with psychological symptoms.

	Paroxetine ($n=32$)		Kamishoyosan ($n=35$)	
	Before	6 months	Before	6 months
Total	18.3 (4.9)	12.6 (4.3)*	17.2 (3.8)	13.2 (3.5)*
Psychological	10.4 (3.3)	7.3 (3.1)*	9.6 (3.1)	7.2 (2.5)*
Anxiety	5.1 (2.2)	4.3 (2.0)*	5.2 (1.9)	4.4 (1.7)*
Depression	5.3 (2.2)	3.3 (1.8)*	4.4 (2.3)	2.8 (1.5)*
Somatic	4.4 (2.1)	3.4 (1.5)*	4.4 (1.9)	3.7 (1.8)*
Vasomotor	2.3 (1.9)	1.0 (0.9)*	2.5 (1.5)	1.5 (1.2)*

Values are means (standard deviations).
* $p<0.0001$ vs. before treatment.

Table 3
Percentage changes in Greene's scores at 6 months after treatments with paroxetine and kamishoyosan.

	Paroxetine ($n=32$)	Kamishoyosan ($n=35$)	*p* values
ΔTotal (%)	−33.0 (13.0)	−22.2 (8.9)	0.0002
ΔPsychological (%)	−34.3 (17.0)	−22.1 (10.1)	0.0007
ΔSomatic (%)	−23.8 (21.2)	−16.4 (20.5)	0.167
ΔVasomotor (%)	−52.3 (33.6)	−33.7 (32.4)	0.05

Values are means (standard deviations). (Δ) percentage change.

($p<0.0001$) decreased (from 18.3 ± 4.9 to 12.6 ± 4.3). Greene's psychological, somatic and vasomotor scores in women treated with paroxetine were also decreased significantly ($p<0.0001$). On the other hand, Greene's total score in women treated with kamishoyosan was decreased (from 17.2 ± 3.8 to 13.2 ± 3.5) significantly ($p<0.0001$). Greene's psychological, somatic and vasomotor scores in women treated with kamishoyosan were also decreased significantly ($p<0.0001$). As shown in Table 3, percentage decreases in total, psychological and vasomotor scores during the 6-month period in the paroxetine group were significantly ($p=0.0002$, 0.0007 and 0.05, respectively) greater than those in the kamishoyosan group.

3.3. Changes in serum cytokine concentrations in women treated with paroxetine and kamishoyosan

There were no significant differences in serum cytokine concentrations in women before treatments with paroxetine and kamishoyosan. As can be seen in Fig. 1, median IL-6 concentration in women treated with paroxetine was decreased (baseline, 1.41 pg/ml; 6 months, 0.55 pg/ml) significantly ($p=0.0003$). Serum concentrations of IL-8, MIP-1β and MCP-1 in women treated with paroxetine was decreased significantly ($p=0.018$, 0.033 and

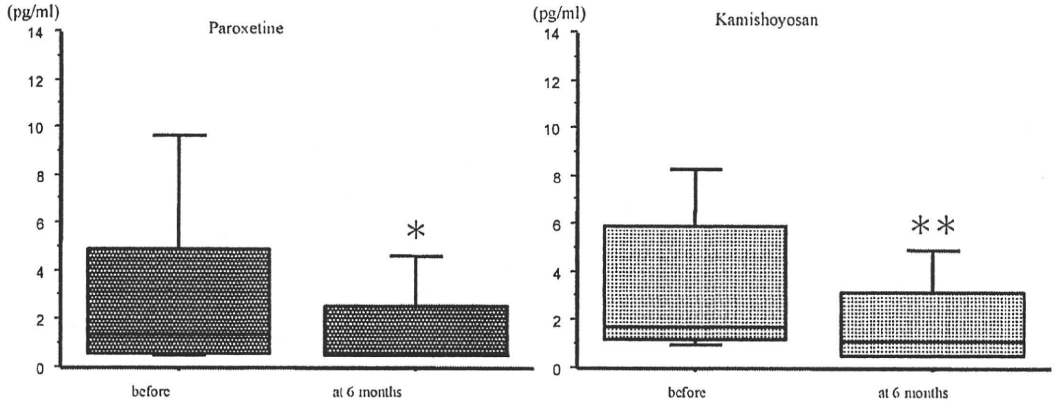

Fig. 1. Changes in serum IL-6 concentrations in women treated with paroxetine and kamishoyosan. Horizontal small bars represented the 10th–90th percentile range, and boxes indicate the 25th–75th percentile range. The horizontal line in each box corresponds to the median; *$p<0.01$, **$p<0.05$.

Table 4
Serum cytokine concentrations before and at 6 months after treatments with paroxetine and kamishoyosan in women with psychological symptoms.

	Paroxetine (n = 32)		Kamishoyosan (n = 35)	
	Before	6 months	Before	6 months
IL-1β (pg/ml)	0.40 (0.40–1.92)	0.40 (0.40–1.42)	0.80 (0.80–1.62)	0.40 (0.40–6.81)
IL-2 (pg/ml)	0.55 (0.55–5.53)	0.55 (0.55–5.46)	0.55 (0.55–3.89)	0.55 (0.55–0.96)
IL-4 (pg/ml)	0.25 (0.25–0.25)	0.25 (0.25–0.25)	0.25 (0.25–0.25)	0.25 (0.25–0.25)
IL-5 (pg/ml)	0.40 (0.40–0.45)	0.40 (0.40–0.49)	0.40 (0.40–0.76)	0.40 (0.40–0.56)
IL-6 (pg/ml)	1.41 (0.55–9.67)	0.55 (0.55–4.68)*	1.73 (1.01–8.35)	1.16 (0.55–4.97)**
IL-7 (pg/ml)	3.59 (0.89–7.14)	3.05 (1.25–4.96)	3.35 (1.29–9.05)	3.09 (0.55–6.36)
IL-8 (pg/ml)	44.3 (5.67–212.8)	18.5 (4.89–102.9)**	46.4 (5.96–216.5)	26.4 (6.75–198.5)
IL-10 (pg/ml)	0.73 (0.45–1.92)	0.45 (0.45–1.32)	0.45 (0.45–1.23)	0.45 (0.45–0.94)
TNF-α (pg/ml)	1.50 (1.50–7.32)	1.50 (1.50–7.57)	1.50 (1.50–8.30)	1.50 (1.50–8.72)
IFN-γ (pg/ml)	9.65 (9.65–9.65)	9.65 (9.65–9.65)	9.65 (9.65–9.65)	9.65 (9.65–9.65)
MCP-1 (pg/ml)	50.1 (20.9–104.5)	38.2 (15.8–66.4)**	45.9 (23.0–90.4)	45.1 (24.0–75.3)
MIP-1β (pg/ml)	195.0 (77.4–449.6)	158.3 (67.4–245.0)**	232.9 (59.2–486.7)	221.7 (76.7–353.9)

Values are medians (10–90 percentiles). IL: interleukin; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ; MCP-1: monocyte chemoattractant protein-1; MIP-1β: macrophage inflammatory protein-1β.
* $p < 0.01$ vs. before treatment.
** $p < 0.05$ vs. before treatment.

0.014, respectively) and serum IL-10 concentrations tended to be decreased ($p = 0.093$) (Table 4). Serum concentrations of TNF-α and IL-1β did not change significantly. On the other hand, median IL-6 concentration in women treated with kamishoyosan was decreased (baseline, 1.73 pg/ml; 6 months, 1.16 pg/ml) significantly ($p = 0.021$), but other serum cytokines and chemokines concentrations did not change significantly.

3.4. Correlations of Greene's scores and serum cytokine concentrations

As can be seen in Fig. 2, IL-6 levels showed significant positive correlations with Greene's total scores in women treated with paroxetine and kamishoyosan, respectively ($r = 0.380$, $p = 0.0013$; $r = 0.273$, $p = 0.018$). In addition, IL-8 and MIP-1β showed significant positive correlations with Greene's total scores, respectively ($r = 0.455$, $p < 0.0001$; $r = 0.329$, $p = 0.0058$), and IL-10 showed a weak correlation with Greene's total score ($r = 0.267$, $p = 0.027$) in women treated with paroxetine (Fig. 3).

3.5. Changes in serum cytokine concentrations in women in whom hot flashes were improved by kamishoyosan and in women in whom kamishoyosan had no effect on hot flashes

We assessed severity of hot flashes using the Food and Drug Administration published draft guidance for clinical evaluation of vasomotor symptoms [18]. Severity is defined as mild (sensation of heat without sweating), moderate (sensation of heat with sweating, able to continue activity), and severe (sensation of heat with sweating, causing cessation of activity). We divided the subjects into two groups: those in whom hot flashes were improved by kamishoyosan as responders (i.e. from severe to mild or moderate) and those in whom kamishoyosan had no effect on hot flashes as non-responders (i.e. from moderate to moderate or severe). As can be seen in Table 5, total, psychological and somatic scores were significantly decreased in both responders and non-responders. In the responder group, serum concentrations of IL-6, IL-8 and MIP-1β were decreased significantly ($p = 0.049$, 0.018 and 0.044, respectively). However, serum IL-8 level in the non-responder group was increased significantly ($p = 0.026$).

3.6. Three-dimensional HPLC profile of kamishoyosan

Three-dimensional HPLC profiles of the methanol solution and the alkaloid fraction of kamishoyosan are shown in Fig. 4.

4. Discussion

In the present study, we showed that serum IL-6 concentrations were decreased by both treatments with paroxetine and kamishoyosan in women with psychological symptoms. Circulating IL-6 concentration has been reported to be significantly high in subjects with major depression and in midlife women with depressive mood [6,7,9,10]. It has been reported that elevated IL-6 concentration was decreased following successful treatment of major depression with fluoxetine [11]. Lanquillon et al. reported

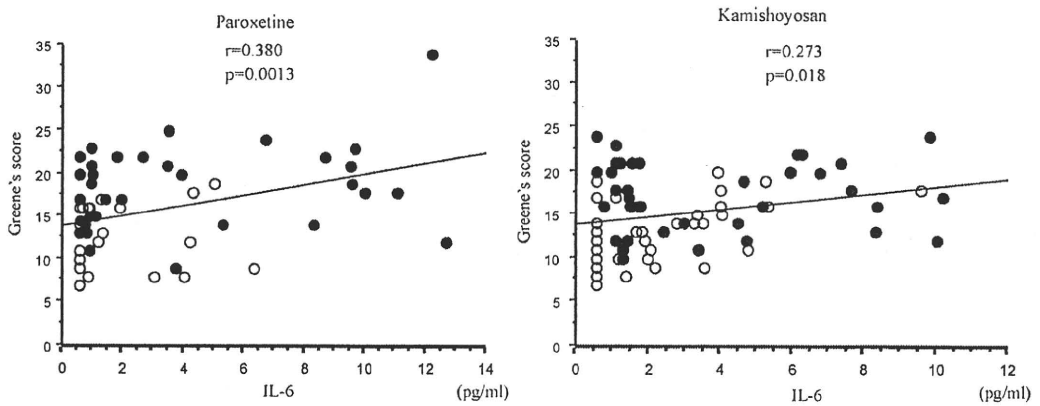

Fig. 2. Correlations of Greene's total scores and serum IL-6 levels in women treated with paroxetine and kamishoyosan: (●) pre-treatment; (○) post-treatment.

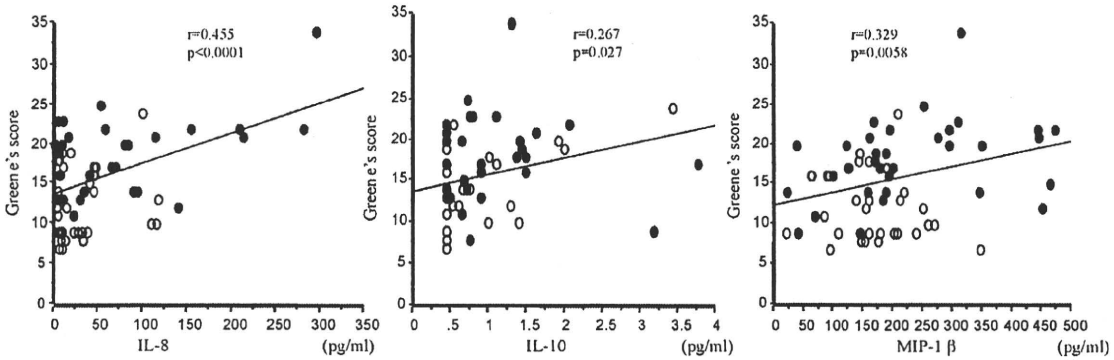

Fig. 3. Correlations of Greene's total scores and serum levels of IL-8, IL-10 and MIP-1β in women treated with paroxetine: (●) pre-treatment; (○) post-treatment.

that IL-6 level decreased in responders to antidepressant treatment but remained high in non-responders [19]. It has also been reported that IL-6 level was decreased significantly after treatment with SSRI [17] and that suppression of IL-6 level was not observed in depressed patients who failed to respond to SSRIs [20]. On the other hand, Ushiroyama et al. reported that there was a decrease in plasma IL-6 level as well as improvement of menopausal symptoms after treatment with kamishoyosan [2]. Therefore, IL-6, which is involved in the pathogenesis of depression, is decreased in response to treatment with paroxetine, and kamishoyosan may also have an effect that is related to the decrease in IL-6 on psychological symptoms.

The mechanism underlying the decrease in IL-6 in response to paroxetine or kamishoyosan is not clear because the source of IL-6 is not fully understood. In the present study, we showed the significant correlations of Greene's scores and IL-6 levels in women treated with paroxetine and kamishoyosan. IL-6 may be involved in the mechanism by which Greene's scores are reduced by treatments of paroxetine and kamishoyosan. IL-6 has been reported to stimulate the hypothalamic-pituitary-adrenocortical (HPA) axis and the release of corticotropin-releasing factor [21]. It has also been reported that IL-6 was produced and released from the rat adrenal zona glomerulosa by stimulation with corticotropin [22]. In addition, disruption of glucocorticoid-mediated feedback inhibition of IL-6 production has been reported in patients with depression [23]. Therefore, paroxetine and kamishoyosan may have effects on the HPA axis and feedback inhibition in women with psychological symptoms.

We found that serum concentrations of IL-8 and MIP-1β were decreased significantly and that both levels showed significant positive correlations with Greene's scores in women treated with paroxetine. We reported previously that serum concentrations of IL-8 and MIP-1β in midlife women with hot flashes were higher than those in midlife women without hot flashes [24]. In addition, we reported that cytokine-induced neutrophil chemoattractant (CINC), which corresponds to IL-8 in humans, was produced in the hypothalamus and might be involved in the pathoetiology of hot flashes [25]. It has been reported that a substantial reduction in hot flashes was observed following paroxetine treatment in menopausal women [26]. Therefore, paroxetine may improve hot flashes due to suppression of the production of IL-8 in the hypothalamus. In the present study, serum concentrations of IL-8 and MIP-1β decreased significantly in women whose hot flashes were improved by kamishoyosan. Kamishoyosan may also reduce concentrations of IL-8 and MIP-1β by acting on the hypothalamus in women whose hot flashes have been improved.

On the other hand, the change in IL-10 in depression is controversial. IL-10 has been shown to be a negative immunoregulatory

Table 5
Greene's scores and serum cytokine concentrations before and at 6 months after treatments with kamishoyosan in non-responders and responders for hot flashes.

	Non-responders for hot flashes ($n = 10$)		Responders for hot flashes ($n = 25$)	
	Before	6 months	Before	6 months
Greene's score				
Total	18.7 (3.3)	15.6 (3.6)**	16.6 (3.8)	12.3 (3.1)*
Psychological	10.4 (3.0)	7.7 (2.3)**	9.2 (3.2)	7.0 (2.6)*
Somatic	5.4 (1.1)	4.6 (1.5)#	4.0 (2.0)	3.4 (1.8)**
Vasomotor	2.2 (1.4)	2.3 (1.4)	2.6 (1.7)	1.2 (0.8)*
IL-1β (pg/ml)	0.80 (0.80–2.19)	0.40 (0.40–7.67)	0.80 (0.80–1.62)	0.40 (0.45–6.06)
IL-2 (pg/ml)	0.55 (0.55–1.75)	0.55 (0.55–1.06)	0.55 (0.55–5.90)	0.55 (0.55–3.42)
IL-4 (pg/ml)	0.25 (0.25–0.25)	0.25 (0.25–0.25)	0.25 (0.25–0.25)	0.25 (0.25–0.25)
IL-5 (pg/ml)	0.40 (0.40–1.00)	0.41 (0.40–0.62)	0.40 (0.40–0.76)	0.40 (0.40–0.49)
IL-6 (pg/ml)	3.01 (1.08–8.62)	2.44 (0.55–5.01)	1.85 (1.01–8.35)	0.55 (0.55–4.57)#
IL-7 (pg/ml)	3.53 (2.18–8.39)	3.05 (1.98–7.15)	3.29 (1.08–9.50)	3.09 (0.10–6.36)
IL-8 (pg/ml)	24.7 (3.75–158.5)	89.1 (9.66–283.5)#	51.7 (6.06–242.5)	22.1 (6.73–98.1)#
IL-10 (pg/ml)	0.45 (0.45–0.46)	0.50 (0.45–0.93)	0.45 (0.45–1.50)	0.45 (0.45–2.11)
TNF-α (pg/ml)	1.50 (1.50–4.39)	1.50 (1.50–2.78)	1.50 (1.50–8.58)	1.50 (1.50–9.52)
IFN-γ (pg/ml)	9.65 (9.65–9.65)	9.65 (9.65–9.65)	9.65 (9.65–9.65)	9.65 (9.65–9.65)
MCP-1 (pg/ml)	50.4 (29.2–81.0)	56.6 (23.1–96.4)	45.7 (21.6–93.3)	45.1 (25.1–75.3)
MIP-1β (pg/ml)	227.8 (37.4–432.1)	251.6 (51.1–388.8)	230.0 (82.4–534.2)	174.7 (102.9–327.7)#

Values in Greene's scores are means (standard deviations). Values in cytokines are medians (10–90 percentiles). IL: interleukin; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ; MCP-1: monocyte chemoattractant protein-1; MIP-1β: macrophage inflammatory protein-β.

* $p < 0.0001$ vs. before treatment.

** $p < 0.001$ vs. before treatment.

\# $p < 0.05$ vs. before treatment.

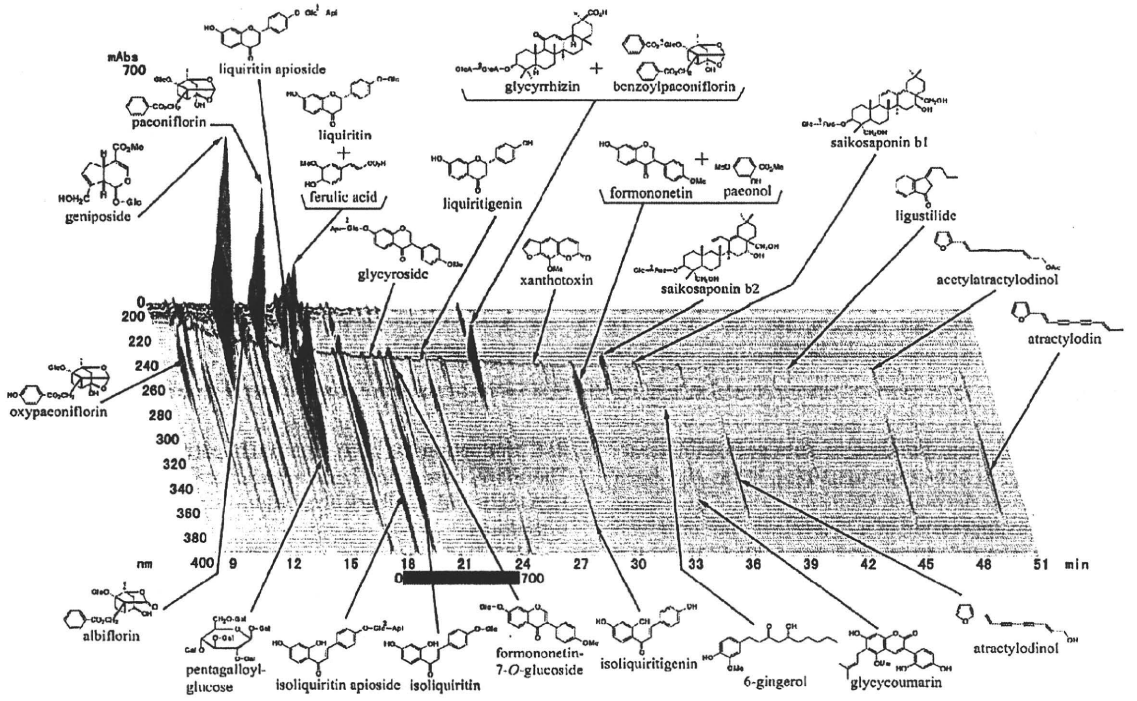

Fig. 4. Three-dimensional HPLC profile of the alkaloid of kamishoyosan.

cytokine [27]. It has been shown that venlafaxine significantly enhances IL-10 production in vitro [28]. However, Seidel et al. demonstrated by using a whole blood cell assay that the production level of IL-10 was elevated in patients with major depressive disorder compared to that in healthy subjects [8]. We also reported that serum IL-10 concentration in women with psychological symptoms was higher than that in women without psychological symptoms [10]. It has recently been reported that IL-10 receptor is expressed in the mouse adrenal gland and that IL-10 plays an important role in the regulation of steroid biosynthesis and in the maintenance of homeostasis and immunity during a period of stress [29]. Further study on the site where paroxetine acts is needed.

In the present study, we found that paroxetine reduced serum MCP-1 concentration. MCP-1 is the primary chemokine responsible for the recruitment of monocytes to sites of active inflammation, including the developing atheroma [30]. The expression of MCP-1 has been reported to be enhanced in endothelial cells [31] and white adipose tissue [32]. Recently, SSRI therapy has been reported to be associated with significant reduction in pro-inflammatory markers [33]. Paroxetine may play a suppressive role in inflammation.

It has been reported that there was a significant decrease in plasma TNF-α after SSRI treatment [12]. Denys et al. demonstrated that SSRI might decrease TNF-α due to activation of the 5-HT_{2A} receptor and increase in 5-HT since 5-HT might inhibit TNF-α [34]. We did not find a significant change in serum TNF-α level in women treated with paroxetine or kamishoyosan. The difference in these results may be due to the difference in subjects.

In the present study, kamishoyosan as well as paroxetine significantly improved psychological symptoms, although the magnitude of the effect of kamishoyosan was weak. On the other hand, compliance in women treated with paroxetine was rather poor due to adverse reactions, such as nausea and headache, but only one woman treated with kamishoyosan dropped out of the study because of adverse reaction. Thus, kamishoyosan may be a candidate for treatment of psychological symptoms because treatment for psychological symptoms must be continued without adverse reactions as long as possible.

It has been reported that kamishoyosan increased social interaction time and that its anxiolytic effect was as strong as that of diazepam [35]. In addition, it has been shown that the anxiolytic effect of kamishoyosan is due to neurosteroid synthesis followed by stimulation of γ-amino-butyric acid_A/benzodiazepine receptor [35]. Recently, many components have been shown to be included in Japanese traditional medicines by three-dimensional HPLC fingerprint analysis as shown for kamishoyosan in Fig. 4. Toriizuka et al. reported that Gardeniae fruit's major component, geniposide, increased social interaction time in a dose-dependent manner [36]. It has been reported that ligustilide and butylidenephthalide, components of Angelica root, reversed the decrease in pentobarbital sleep in mice [37]. Further study is needed to clarify which components of kamishoyosan have effects on cytokines and whether these components have effects similar to those of paroxetine.

In conclusion, decrease in IL-6 concentration may be involved in the mechanism of the actions of both paroxetine and kamishoyosan in women with psychological symptoms, and IL-6 may therefore be useful as a marker of treatment. In addition, the action of paroxetine may be associated with decrease in other cytokines and chemokines, including IL-8, IL-10, MIP-1β.

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ORIGINAL PAPER

Association Study Between the Pericentrin (*PCNT*) Gene and Schizophrenia

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Abstract Disrupted-in-schizophrenia 1 (*DISC1*), a known genetic risk factor for schizophrenia (SZ) and major depressive disorder (MDD), interacts with several proteins and some of them are reported to be genetically associated with SZ. Pericentrin (*PCNT*) also interacts with *DISC1* and recently single-nucleotide polymorphisms (SNPs) within the *PCNT* gene have been found to show significant associations with SZ and MDD. In this study, case-controlled association analysis was performed to determine if the *PCNT* gene is implicated in SZ. Nine SNPs were analyzed in 1,477 individuals (726 patients with SZ and 751 healthy controls). No significant difference was observed between the controls and the patients in allelic frequencies or genotypic distributions of eight SNPs. Although allelic distribution of rs11702684 was different between the two groups ($P = 0.042$), the difference did not reach statistical significance after permutation correction for multiple comparisons. In the haplotypic analysis, we could not find any significant association in our subjects, either. This gene may not play a major role independently in the etiology of SZ in the Japanese population.

Keywords Schizophrenia · PCNT · Kendrin · Case–control association study · DISC1

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Introduction

Schizophrenia (SZ) is a complex psychiatric disorder that afflicts approximately 1% of the population throughout the world and has high heritability (Craddock et al. 2005). The pericentrin gene (the official symbol; *PCNT* and also called kendrin) is located at 21q22.3, which is one of chromosomal lesions prevalent in SZ by cytogenetic analysis (Demirhan and Tastemir 2003). *PCNT* is a coiled-coil protein localized specifically to the centrosome throughout the cell cycle (Flory et al. 2000) and an integral component of the pericentriolar material (Li et al. 2001). This protein provides sites for microtubule nucleation in the centrosome by anchoring gamma-tubulin complex (Takahashi et al. 2002), then it plays an important role in microtubule organization, spindle organization, and chromosome segregation (Doxsey et al. 1994; Purohit et al. 1999). Disrupted-in-schizophrenia 1

(*DISC1*), a known genetic risk factor for SZ and major depressive disorder (MDD) (Cannon et al. 2005; Chen et al. 2007; Hashimoto et al. 2006; Hennah et al. 2003; Millar et al. 2000; Thomson et al. 2005), localizes to the centrosome by binding to *PCNT* (Miyoshi et al. 2004). Shimizu et al. showed that overexpression of the *DISC1*-binding regions of *PCNT* or the *DISC1* deletion mutant lacking the *PCNT*-binding region impaired the microtubule organization and they suggested that the *DISC1–PCNT* interaction played a key role in the microtubule network formation (Shimizu et al. 2008). Recently, single-nucleotide polymorphisms (SNPs) within the *PCNT* gene have been found to show allelic associations with SZ and MDD (Anitha et al. 2009; Numata et al. 2009). In addition, Mitkus et al. reported a trend for an increase mRNA levels of the *PCNT* gene in the dorsolateral prefrontal cortex of patients with SZ, compared with the control groups (Mitkus et al. 2006). In this study, case-controlled association analysis was performed in the Japanese population to determine if the *PCNT* gene is implicated in SZ.

Materials and Methods

Subjects

We used genomic DNA samples from 726 SZ patients: 406 male (mean age 48.6 ± 13.8 years), 320 female (mean age 49.2 ± 14.5 years) from the Tokushima University Hospital, affiliated psychiatric hospitals of the University of Tokushima, the Ehime University Hospital and the Osaka University Hospital in Japan. The diagnosis of SZ was made by at least two experienced psychiatrists according to DSM-IV criteria on the basis of extensive clinical interviews and review of medical records. Seven hundred fifty-one controls, 422 male (mean age 45.5 ± 11.1 years) and 329 female (mean age 45.2 ± 10.5), were selected from volunteers who were recruited from hospital staff and students and company employees documented to be free from either psychiatric problems or past mental histories. All subjects were unrelated Japanese origin and signed written informed consent to participate in the genetic association studies approved by the institutional ethics committees.

Genotyping

We initially selected eight tagging SNPs by SNPBrowser 3.5 (De La Vega et al. 2006) (Applied Biosystems, Foster, CA, USA, Pair-wise $r^2 > 85\%$, MAF > 20%, Japanese population) (rs11702684, rs2249057, rs11701058, rs2839226, rs2839231, rs3788265, rs2073376, rs1010111) (Supplementary Table 1). After that, we selected rs2073380 additionally because eight tagging SNPs did not seem to cover the third block of the *PCNT* gene from HapMap data. Genotyping was performed using commercially available TaqMan probes for the *PCNT* gene with ABI Prism 7900 HT Sequence Detection System and ABI 7500 Real Time PCR System (Applied Biosystems). Haplotype block structure was determined using the HAPLOVIEW program (Barrett et al. 2005). Blocks were defined according to the criteria of Gabriel et al. (2002).

Statistical Analysis

Allelic and genotypic frequencies of patients and control subjects were compared using χ^2 test. The SNPAlyze 3.2Pro software (DYNACOM, Japan) was used to estimate haplotype frequencies, linkage disequilibrium (LD), permutation *P*-values (10,000 replications) and deviation from Hardy–Weinberg Equilibrium (HWE) distribution of alleles. Power calculations for our sample size performed using the G*Power program (Erdfelder et al. 1996). The criterion for significance was set at $P < 0.05$ for all tests.

Results

Genotypic and allelic frequencies of the *PCNT* gene are shown in Table 1. Genotypic distributions of these nine SNPs did not deviate significantly from HWE in either group ($P > 0.05$). No significant difference was observed in genotypic frequency between the controls and patients in eight SNPs. Although allelic distribution of rs11702684 was different between the two groups ($P = 0.042$), the difference did not reach statistical significance after permutation correction for multiple comparisons. In power calculations using the G*Power program, our sample size had >0.98 power for detecting a significant association (alpha < 0.05) when an effect size index of 0.2 was used.

Several papers reported that there were gender-specific genetic components involved in the pathology of SZ in the *DISC1* gene (Hennah et al. 2003; Chen et al. 2007) and the *DISC1*-related genes (Hennah et al. 2007; Pickard et al. 2007; Qu et al. 2008). In our study, when the data were subdivided on the basis of gender, allelic distribution of rs11702684 was different between the two groups in only male samples ($P = 0.033$). However, the difference did not survive statistical significance after permutation correction for multiple comparisons.

There were three LD blocks in the *PCNT* gene with rs2249057, rs11701058, rs2839226, and rs2839231 residing in block 1 and rs3788265 and rs2073376 residing in block 2, and rs2073380 and rs1010111 residing in block 3 (Gabriel et al. 2002, Fig. 1). These constructed marker haplotypes of blocks 1–3 were not associated with SZ (permutation $P = 0.184$, 0.137, and 0.601, respectively).

Table 1 Genotypes and allele frequencies of nine single SNPs in the PCNT gene in patients with SZ and controls

SNP	Diagnosis	Allele		*P*-value	Genotype			*P*-value	Frequency
rs11702684		C	T		C/C	C/T	T/T		
	SC	913	515	0.042	296	321	97	0.085	0.361
	CT	892	588		265	362	113		0.397
		C	A		C/C	C/A	A/A		
rs2249057	SC	862	590	0.504	255	352	119	0.691	0.406
	CT	870	626		247	376	125		0.418
		C	T		C/C	C/T	T/T		
rs11701058	SC	669	783	0.181	153	363	210	0.297	0.461
	CT	728	772		168	392	190		0.485
		C	T		C/C	C/T	T/T		
rs2839226	SC	378	1072	0.111	47	284	394	0.19	0.261
	CT	353	1147		34	285	431		0.235
		A	G		A/A	A/G	G/G		
rs2839231	SC	408	1042	0.562	63	282	380	0.52	0.281
	CT	405	1085		53	299	393		0.272
		G	T		G/G	G/T	T/T		
rs3788265	SC	821	627	0.998	234	353	137	0.506	0.433
	CT	846	646		230	386	130		0.433
		A	G		A/A	A/G	G/G		
rs2073376	SC	445	1001	0.403	75	295	353	0.51	0.308
	CT	478	1006		77	324	341		0.322
		C	A		C/C	C/A	A/A		
rs2073380	SC	642	796	0.839	144	354	221	0.552	0.446
	CT	669	817		141	387	215		0.45
		A	G		A/A	A/G	G/G		
rs1010111	SC	1079	363	0.298	402	275	44	0.343	0.252
	CT	1141	351		428	285	33		0.235

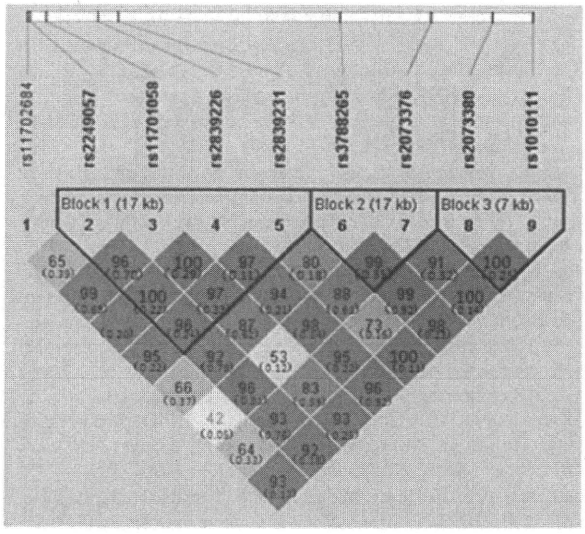

Fig. 1 LD and haplotype structure of the PCNT gene. Haplotype block structure was determined using the HAPLOVIEW program (Barrett et al. 2005). Blocks were defined according to the criteria of Gabriel et al. (2002). Each box represents the D' (r^2) values corresponding to each pair-wise SNP

Discussion

In this study, we examined the association of nine SNPs in the *PCNT* gene and SZ. No significant difference was observed between the controls and the patients in either allelic frequencies or genotypic distributions of nine SNPs after permutation correction for multiple comparisons. In the haplotypic analysis, we could not find any significant association in our subjects. This result was concordance with another study in a Caucasian population (Tomppo et al. 2009).

During the preparation of this article, Anitha et al. reported that rs2249057 of the *PCNT* gene and haplotypes involving this SNP were significantly associated with SZ after correction for multiple comparisons in the Japanese population (Anitha et al. 2009). Although SNPs examined in our study contained rs2249057, we could not find any significant associations in our subjects. The statistical power of our study was sufficient to detect an association between the variants and SZ (SZ $n = 726$; control $n = 751$). Surprisingly, the control minor allele frequency of rs2249057 in Anitha's study (0.48) was higher than

those of our study, HapMap data, and ABI data (0.42, 0.40, and 0.41, respectively). This differing allele frequency between these two studies may be caused by samples' recruited areas. Anitha et al. used subjects from further east compared to ours. However, it is reported that there is no significant population stratification in Japanese (Arinami et al. 2005; Yamaguchi-Kabata et al. 2008).

There are several limitations in our study. First, we applied MAF > 20% when we selected the tagging SNPs and it is difficult to evaluate the association of rare variants in our study. Second, we cannot rule out a possibility that *DISC1–PCNT* interaction may be involved in the etiology of SZ. Third, our findings only represented the Japanese population and studies in other populations would still be warranted due to differing allele frequencies between populations.

Conclusions

In conclusion, we did not find any significant association between the *PCNT* gene and the SZ in the Japanese population. This gene may not play a major role independently in the etiology of SZ.

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GABA concentration in schizophrenia patients and the effects of antipsychotic medication: A proton magnetic resonance spectroscopy study

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ABSTRACT

Gamma-amino butyric acid (GABA) is thought to play a role in the pathophysiology of schizophrenia. High magnetic field proton magnetic resonance spectroscopy (^{1}H-MRS) provides a reliable measurement of GABA in specific regions of the brain. This study measured GABA concentration in the anterior cingulate cortex (ACC) and in the left basal ganglia (ltBG) in 38 patients with chronic schizophrenia and 29 healthy control subjects.
There was no significant difference in GABA concentration between the schizophrenia patients and the healthy controls in either the ACC (1.36 ± 0.45 mmol/l in schizophrenia patients and 1.52 ± 0.54 mmol/l in control subjects) or the ltBG (1.13 ± 0.26 mmol/l in schizophrenia patients and 1.18 ± 0.20 mmol/l in control subjects). Among the right handed schizophrenia patients, the GABA concentration in the ltBG was significantly higher in patients taking typical antipsychotics (1.25 ± 0.24 mmol/l) than in those taking atypical antipsychotics (1.03 ± 0.24 mmol/l, $p = 0.026$). In the ACC, the GABA concentration was negatively correlated with the dose of the antipsychotics ($rs = -0.347$, $p = 0.035$). In the ltBG, the GABA concentration was positively correlated with the dose of the anticholinergics ($rs = 0.403$, $p = 0.015$).
To the best of our knowledge, this is the first study to have directly measured GABA concentrations in schizophrenia patients using ^{1}H-MRS. Our results suggest that there are no differences in GABA concentrations in the ACC or the ltBG of schizophrenia patients compared to healthy controls. Antipsychotic medication may cause changes in GABA concentration, and atypical and typical antipsychotics may have differing effects. It is possible that medication effects conceal inherent differences in GABA concentrations between schizophrenia patients and healthy controls.

1. Introduction

1.1. The Gamma-amino butyric acid (GABA) system in schizophrenia

Gamma-amino butyric acid (GABA) is thought to play a role in the pathophysiology of schizophrenia (Guidotti et al., 2005; Wassef et al., 2003).

1.1.1. Postmortem studies

Postmortem studies of $GABA_A$ receptors in chronic schizophrenia have reported inconsistent findings. Some

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case–control studies have reported increased $GABA_A$ receptor binding in the cingulate cortex (Hanada et al., 1987; Benes et al., 1992), whereas others have found it to be decreased (Squires et al., 1993) or unchanged (Pandey et al., 1997). The $GABA_A$ receptor is composed of various subunits. Ishikawa et al. (2004) found a higher density of alpha 1 and beta 2/3 subunits in the prefrontal cortex (PFC) of schizophrenia patients compared to control subjects.

The 65 and 67 kDa isoforms of glutamic acid decarboxylase (GAD) are key enzymes in GABA synthesis, and a number of studies have investigated their significance in schizophrenia. Bird et al. (1977) found that GAD levels were decreased in the nucleus accumbuns, amygdala, hippocampus, and putamen of schizophrenia patients. Benes et al. (2000) observed no change in GAD density in the anterior cingulate cortex (ACC) of schizophrenia patients. Woo et al. (2004) found a decrease in the number of GAD67 mRNA-containing neurons in the ACC of schizophrenia patients compared to control subjects. Dracheva et al. (2004) reported an increased expression of GAD65 and GAD67 mRNA in the dorsolateral PFC and in the occipital cortex of schizophrenia patients compared to control subjects.

1.1.2. In vivo neuroimaging studies

Neuroimaging studies using radio active ligands have also reported inconsistent findings. Some studies using single photon emission computed tomography (SPECT) have failed to find any evidence of $GABA_A$ receptor binding abnormalities in the brains of schizophrenia patients compared to healthy controls (Busatto et al., 1997; Verhoeff et al., 1999; Abi-Dargham et al., 1999). One study, however, found a significant correlation between task performance and $GABA_A$/benzodiazepine receptor binding in the frontal and occipital cortices of schizophrenia patients (Ball et al., 1998). Using positron emission tomography (PET), Asai et al. (2008) reported no differences in [11C] Rol5-4513 binding (which represents the density of the alpha 5 subunit of the $GABA_A$/benzodiazepine receptor) in the PFC and the hippocampus of schizophrenia patients compared to control subjects; among the schizophrenia patients, the degree of binding was found to be negatively correlated with negative symptom scores.

1.1.3. The effects of antipsychotic medication on the GABA system in the basal ganglia and cingulate cortex

Gunne et al. (1984) reported an inhibition of GAD activity in monkeys following treatment with antipsychotics. Studies in rats have reported that treatment with typical antipsychotic drugs such as haloperidol (Jolkkonen et al., 1994; Delfs et al., 1995a,b; Laprade and Soghomonian, 1995; Sakai et al., 2001), fluphenazine (Chen and Weiss, 1993; Johnson et al., 1994), and sulpiride (Laprade and Soghomonian, 1995) increased the expression of GAD67 and GAD67 mRNA in the basal ganglia, whereas atypical antipsychotic drugs such as clozapine (Delfs et al., 1995a) and olanzapine (Sakai et al., 2001) did not. These changes may be reflected in the dyskinetic and antipsychotic actions of typical antipsychotics (Delfs et al., 1995b; Sakai et al., 2001). Zink et al. (2004) reported that both haloperidol and clozapine increased [^{3}H]-muscimol binding to $GABA_A$ receptors in the ACC, whereas increased $GABA_A$ receptor binding in the basal ganglia was only induced by haloperidol. Although the underlying mechanism is unclear, these results suggest that antipsychotics may affect the GABA system, and that typical and atypical antipsychotics may have differing effects.

1.2. The role of the ACC and the basal ganglia in schizophrenia

Several changes in the ACC of schizophrenia patients have been reported: (1) alterations in GAD levels (Woo et al., 2004), (2) morphological change (Baiano et al., 2007; Fujiwara et al., 2007; Zetzsche et al., 2007), and (3) activation deficits during cognitive tasks (Liddle et al., 2006; Yücel et al., 2007; Brüne et al., 2008; Koch et al., 2008). Menzies et al. (2007) found that GABA-modulating drugs affected working memory performance and induced activation changes in the ACC of schizophrenia patients. The basal ganglia contain the striatum, the globus pallidus, and other structures. The striatum is thought to receive GABAergic interneurons from other regions of the brain, in particular the globus pallidus and the cerebral cortex (Bolam et al., 2000). The PFC is thought to be involved in the pathophysiology of schizophrenia on three levels: morphologically (Meda et al., 2008), functionally (Lee et al., 2006), and histologically (Woo et al., 2008). The PFC tonically inhibits striatal dopamine projections, and it is thought that this is mediated by GABA interneurons (Carlsson, 2001; Akil et al., 2003; Perlman et al., 2004). The globus pallidus is also thought to be involved in the pathophysiology of schizophrenia (Galeno et al., 2004; Spinks et al., 2005). An increase in $GABA_A$ receptor binding in the basal ganglia following the administration of antipsychotics has been reported (Zink et al., 2004).

1.3. Magnetic resonance spectroscopy

Proton magnetic resonance spectroscopy (^{1}H-MRS) provides an in vivo measurement of brain metabolites such as myo-inositol, N-acetylaspartate, choline-containing compounds, Glx (glutamate plus glutamine), creatine, and phosphocreatine in the human brain. The recent introduction of high magnetic field MRS has enabled the reliable measurement of GABA in specific brain regions. Reduced concentrations of GABA in depressed patients (Hasler et al., 2007) and unchanged concentrations of GABA in panic disorder patients (Hasler et al., 2008) have been reported in areas of the frontal lobe. To the best of our knowledge, no previous ^{1}H-MRS study has examined GABA concentrations in schizophrenia patients. In the present study, ^{1}H-MRS was used to compare GABA concentrations in medicated chronic schizophrenia patients with those of healthy controls using a high magnetic field device. The regions of interest (ROIs) were located in the ACC and in the left basal ganglia (ltBG); the ltBG contain the striatum, globus pallidus, and other structures. The effects of typical and atypical antipsychotic medication on GABA concentrations in the basal ganglia and cingulate cortex were also examined.

2. Method

2.1. Subjects

Thirty-eight patients with chronic schizophrenia and twenty-nine healthy control subjects participated in this

study. All study participants gave written informed consent in accordance with the guidelines of the ethics committee of the University of Tokushima. All diagnoses were assigned according to DSM-IV TR criteria. The schizophrenia patients were classified into the following two groups in order to compare the effects of the type of antipsychotic regime: (1) the TYP group, who were taking typical antipsychotics with or without concomitant atypical antipsychotics, and (2) the ATY group, who were taking atypical antipsychotics without concomitant typical antipsychotics. The patients were also classified according to schizophrenia subtype: paranoid schizophrenia ($n=36$), undifferentiated schizophrenia ($n=1$), and disorganized schizophrenia ($n=1$). Epidemiological data, including age, duration of illness, handedness, Positive and Negative Syndrome Scale scores (PANSS, Kay et al., 1987), and dose of antipsychotics at the time of the scan, are shown in Table 1. The healthy control subjects had no history of any Axis I psychiatric disorder according to DSM-IV TR criteria. None of the patients or healthy control subjects had a serious medical illness, history of head injury, or history of drug or alcohol abuse prior to the scan. All of the schizophrenia patients and healthy control subjects were Japanese and came from the same region, and their native language was Japanese. Handedness was assessed using the Edinburgh Handedness Inventory (Oldfield, 1971). With the exception of two schizophrenia patients, all study participants were right handed.

2.2. The ^{1}H-MRI/MRS procedures

^{1}H-MRS was performed using MEGA-PRESS according to previously reported methods (Mescher et al., 1998). A 3 T clinical magnetic resonance imaging (MRI) scanner was used (Sigma 3T Excite, GE, Milwaukee, WI, USA). Gradients were employed surrounding the frequency selective pulses at 1.9 ppm to diphase transverse magnetization. Water suppression involved three conventional CHESS pulses after manual optimization. The sequence parameters were as follows: (1) TR = 1500 ms, (2) TE = 68 ms, (3) size of ROI = $3.0\times3.0\times3.0$ cm^3 (27 ml), (4) summation = 256 signals for each spectrum, and (5) total acquisition time = 13 min. Alternate measurements with and without frequency selective pulses were taken; J evolution for GABA was refocused during odd-numbered acquisitions but not during even-numbered acquisitions. The difference in the acquired spectra provided an edited spectrum of GABA. The in vitro data for GABA were acquired from MEGA-PRESS, and were used as a basis for the Linear combination model (LC Model) (Provencher, 1993). The quantification of signals from the different MEGA-PRESS spectra was performed with the LC Model. An axial cut approximately 1 cm above the upper end of the body of the lateral ventricles was chosen as a reference slice of the ROI in the ACC. The center of the interhemispheric fissure was 3 cm in front of the central fissure and 2 cm above the corpus callosum (Fig. 1a). An axial cut approximately 1 cm above the genu of the corpus callosum, which provided a continuous view of the anterior and posterior horns of the lateral ventricles, was chosen as a reference slice of the ROI in the ltBG. The ROI of the ltBG was located between the sylvian fissure and the lateral ventricles in order to incorporate the lenticular nucleus (Fig. 1b). The representative ^{1}H-MRS spectra from the MEGA-PRESS sequence are shown in Fig. 2.

T1-weighted three dimensional images were acquired using the following parameters: (1) TE = 4.2 ms, (2) TR = 10 ms, (3) slice thickness = 0.8 mm, (4) matrix 512×512, (5) FOV = 24×24 cm, and (6) Flip angle = 15°. The brain images were thus composed of voxels that were $0.47\text{mm}\times0.47\text{mm}\times0.8\text{mm}$ in size. On the basis of the histogram of voxel intensity, each voxel in the 3D-SPGR brain images was classified as gray matter (GM), white matter (WM), or cerebrospinal fluid (CSF) using the "ImageJ Ver. 1.38" software package (http://rsb.info.nih.gov/ij). The voxels that were considered GM, WM, and CSF in each ROI were counted using the "3D-Slicer Ver.2.6" software package (http://www.slicer.org) in order to obtain the ratio of these tissues. Metabolite concentrations in the ROI were corrected for CSF by dividing the percentage of brain tissues in each ROI, under the assumption that the metabolite concentrations in CSF were equal to zero (Bustillo et al., 2001).

The criteria for selecting the reliable metabolite concentrations were based on the %SD of the fit for each metabolite, reflecting the Cramer–Rao lower bounds (CRLB) for LC Model analysis. The data included in the present study showed %SD of less than 20%. In our previous study, the intraclass correlation of two measurements in the same study participant was greater than 0.7, indicating acceptable reliability as a clinical instrument.

Table 1
Epidemiologic data.

		Schizophrenia	Controls	p
Age	(all)	34.0 ± 10.0	34.0 ± 10.2	n.s.[a]
(mean ± S.D. years old)	(rt handed)	34.7 ± 10.0	34.0 ± 10.2	n.s.[a]
Number (male/female)	(all)	38 (20/18)	29 (17/12)	n.s.[b]
	(rt handed)	36(19/17)	29 (17/12)	n.s.[b]
Duration of illness (years)		11.1 ± 9.4		
PANSS total score (mean ± S.D.)		54.0 ± 14.4		
PANSS positive score (mean ± S.D.)		13.2 ± 6.0		
PANSS negative score (mean ± S.D.)		15.1 ± 5.3		
PANSS general score (mean ± S.D.)		26.7 ± 6.9		
Dose of antipsychotics[c] (mean ± S.D.)		423.7 ± 362.3		

Abbreviation: rt handed, right handed; PANSS, Positive and Negative Syndrome Scale; n.s., not significant.
[a] Two sample Student's *t*-test.
[b] χ square test.
[c] Chlorpromazine equivalent.

2.3. Statistical procedures

Statistical tests were performed using the "SPSS Version 11.5" software package (SPSS Japan Inc., Tokyo, Japan). The absolute GABA levels, as measured with ^{1}H-MRS, were analyzed.

For both ROIs, two sample *t*-tests were used to compare the mean values of the GABA concentrations in schizophrenia patients and healthy control subjects. The GABA concentration of the TYP group was compared to that of the ATY group using analysis of covariance (ANCOVA), with covariance for age.

For both ROIs, the correlation between GABA concentration and each of the clinical measures (e.g., age, dose of benzodiazepine, dose of antipsychotics, dose of anticholinergics, and the positive, negative, and general PANSS scores) was

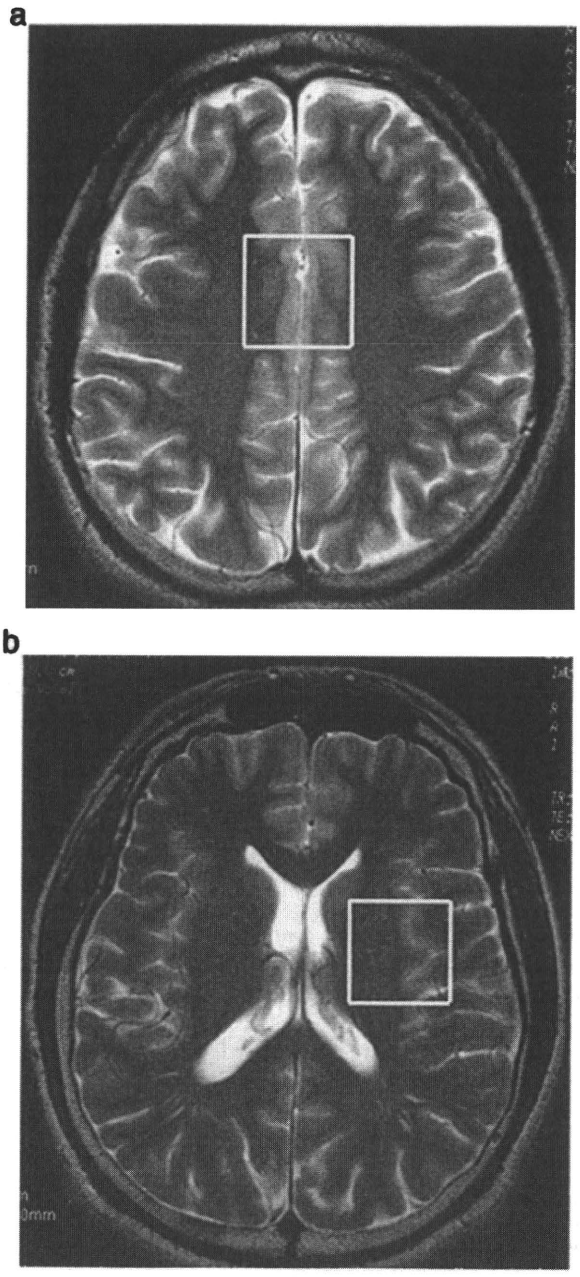

Fig. 1. The region of interest (ROI) positions in the anterior cingulate cortex (ACC) (a) and left basal ganglia (ltBG) (b) for spectroscopic measurement by MEGA-PRESS sequence. The white box represents the location of the ROI (3 cm × 3 cm × 3 cm) that was used in the MEGA-PRESS sequence in the horizontal image.

evaluated using Spearman's rank correlation test. Since handedness could have influenced the results, the analysis in the ltBG was only performed for right handed patients i.e. the two left handed schizophrenia patients were excluded.

Statistical significance was set at $p<0.05$.

Since the partial volume effects of the GM and the WM may influence the GABA concentration in ^{1}H-MRS (Choi et al., 2006), a two sample t-test was used to make the following comparisons of the GM:WM ratio for each ROI: (1) schizophrenia patients and healthy control subjects, and (2) male study participants and female study participants.

3. Results

There were no significant differences between the schizophrenia patients and the healthy control subjects in age range or male:female ratio. No significant difference in GABA concentration was observed between schizophrenia patients and healthy control subjects in either the ACC (1.36 ± 0.45 mmol/l in schizophrenia patients and 1.52 ± 0.54 mmol/l in healthy control subjects, $p=0.18$) or the ltBG (1.13 ± 0.26 mmol/l in schizophrenia patients and 1.18 ± 0.20 mmol/l in healthy control subjects, $p=0.36$).

For the schizophrenia patients, the clinical data of the TYP group and the ATY group are shown in Table 2. The TYP group consisted of 16 patients and the ATY group consisted of 22 patients. For each patient, the name and dose of the antipsychotic medication prescribed at the time of the scan are provided in Tables 3a and b. Eight of the TYP group and 8 of the ATY group were prescribed benzodiazepines, and 13 of the TYP group and 9 of the ATY group were prescribed anticholinergic medication (Tables 3a and b). Three patients were prescribed concomitant mood stabilizers, and a further three patients were prescribed antidepressants (Tables 3a and b). There were no significant differences between the TYP group and the ATY group for the following factors: (1) PANSS positive, negative, and general scores, (2) dose of benzodiazepine (diazepam equivalent dose), and (3) dose of antipsychotics (chlorpromazine equivalent dose). However, age and duration of illness were significantly higher in the TYP group than the ATY group. The TYP group were prescribed anticholinergic medication significantly more often than the ATY group ($X^2=6.18$, $p=0.013$). The mean dose of anticholinergic medication (biperiden equivalent dose) did not differ between the TYP group and the ATY group.

ANCOVA was performed using the type of antipsychotic (i.e. TYP versus ATY) and the use of anticholinergic medication (i.e. patients with versus patients without anticholinergic medication) as two independent factors and with age as a covariate. In the ltBG, ANCOVA revealed a significant effect for the type of antipsychotic ($F=5.48$, $p=0.026$) but not for the use of anticholinergic medication; no interaction between the type of antipsychotic and the use of anticholinergic medication was observed. In the ACC, no significant effect was observed for the type of antipsychotic or the use of anticholinergic medication, and no interaction between these two factors was observed. In the ltBG, the mean ± SD of the GABA concentrations for the TYP group and the ATY group were 1.25 ± 0.24 mmol/l and 1.03 ± 0.24 mmol/l, respectively. In the ACC, the mean ± SD of the GABA concentrations for the TYP group and the ATY group were 1.40 ± 0.48 mmol/l and 1.27 ± 0.51 mmol/l, respectively.

A significant negative correlation was found between the dose of the antipsychotics and the GABA concentration in the ACC ($rs=-0.387$, $p=0.016$) but not in the ltBG. A significant positive correlation was found between the dose of the anticholinergic medication and the GABA concentration in the ltBG ($rs=0.399$, $p=0.016$) but not in the ACC. There was no correlation between the remaining clinical measures (i.e.

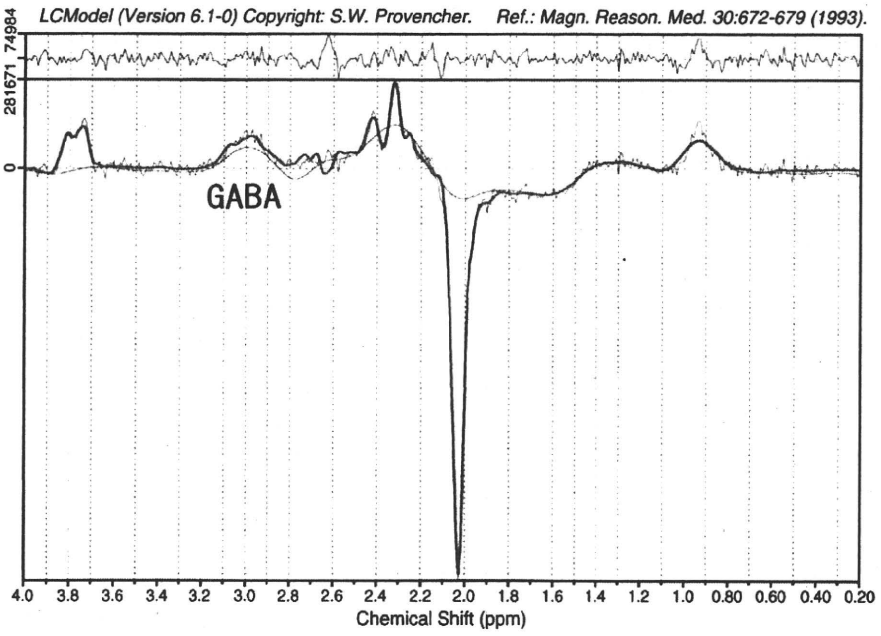

Fig. 2. Representative ^{1}H-MRS spectra from a study participant before the MEGA-PRESS sequence. The peaks that represent GABA are shown.

age, PANSS scores, and dose of benzodiazepine) and the GABA concentrations in the ACC and the ltBG.

The GM:WM ratio did not differ significantly between the schizophrenia patients and the healthy control subjects, or between the TYP group and the ATY group in either of the two ROIs (Table 4).

4. Discussion

4.1. Main findings

This study contributes four main findings. Firstly, no difference in GABA concentration was observed between the schizophrenia patients and the healthy control subjects. Secondly, among the schizophrenia patients, the GABA concentration in the ltBG was significantly higher in the TYP group than in the ATY group. Thirdly, there was a significant negative correlation between the GABA concentration in the ACC and the dose of antipsychotics. Finally, there was a positive correlation between the GABA concentration in the ltBG and the dose of anticholinergic medication.

4.2. No difference in the GABA concentration between schizophrenia patients and healthy control subjects

Analysis of GABA receptor binding sites and measurement of GAD have frequently been used to investigate the GABA

Table 2
The clinical indices of the schizophrenic patients and types of medication.

		TYP	ATY	p
Number	(all)	16	22	
	(rt handed)	15	21	
Age	(all)	38.4±10.7	30.8±8.3	.025*
	(rt handed)	38.7±11.1	31.6±8.2	.046*
Duration of illness	(all)	16.2±11.0	7.1±6.2	.005*
	(rt handed)	16.2±11.0	7.2±6.5	.010*
PANSS positive		12.4±2.6	13.7±7.5	n.s.
PANSS negative		15.0±4.0	15.2±6.1	n.s.
PANSS general		27.0±6.3	27.0±7.3	n.s.
Dose of antipsychotics		435.9±432.9	414.8±311.7	n.s.
Dose of benzodiazepine		6.6±8.3	3.3±4.7	n.s.

Abbreviation: TYP, patients taking typical antipsychotics with or without concomitant atypical antipsychotics; ATY, patients taking atypical antipsychotics without concomitant typical antipsychotics; rt handed, right handed; PANSS, Positive and negative syndrome scale.
* Significantly different in two sample *t*-test ($p<0.05$).

Table 3a
Typical antipsychotic group.

Age (years)	Sex	Antipsychotic drugs	BZD	Anticholin
40	F	HPD 0.75 mg	0	0
30	F	HPD 3 mg	0	3
50	F	HPD 1.5 mg	0	3
36	M	HPD 3 mg	0	3
26	F	HPD 2.25 mg	10	2
48	F	HPD 4 mg	10	2
54	M	HPD 2.25 mg	0	6
46	F	SPD 150 mg	10	0
24	F	OLZ 10 mg, HPD 4.5 mg	30	4
39	M	OLZ 30 mg, LP 100 mg	10	3
21	F	PER 16 mg, BPD 1.5 mg	0	2
33	M	QTP 400 mg, RIS 6 mg, LP 150mg	10	4
24	M	RIS 2 mg, LP 25 mg	0	1
36	M	RIS 3 mg, CP 100 mg, LP 100 mg	15	1
52	M	RIS 6 mg, BPD 6 mg	0	3
46	M	RIS 9 mg, LP 50 mg, ZTP 75 mg	10	0

Abbreviations: BZD, benzodiazepine dose expressed as diazepam equivalent dose; Anticholin, anticholinergic drugs expressed as biperiden equivalent dose; HPD, haloperidol; SPD, sulpiride; LP, levomepromadine; BPD, bromperidol; CP, chlorpromadine; ZTP, zotepin.

Table 3b
Atypical antipsychotic group.

Age (years)	Sex	Antipsychotic drugs	BZD	Anticholin
16	F	APZ 6 mg	0	0
25	M	APZ 12 mg	0	0
37	M	APZ 12 mg	10	1
30	F	APZ 6 mg, OLZ 2.5 mg	0	0
21	F	APZ 6 mg, RIS 1 mg	10	1
27	M	OLZ 5 mg	10	0
25	M	OLZ 5 mg	0	0
35	M	OLZ 15 mg	0	0
40	M	OLZ 20 mg	5	0
20	F	OLZ 20 mg	10	0
21	M	OLZ 25 mg	6.7	1
38	F	OLZ 25 mg	0	0
27	M	PER 2 mg	0	0
28	F	PER 16 mg	8.33	0
38	F	PER 20 mg	0	2
28	M	QTP 750 mg	0	2
25	F	RIS 2 mg	0	2
37	F	RIS 2 mg	0	0
36	M	RIS 2 mg	0	0
48	M	RIS 5 mg	0	1
25	F	RIS 8 mg	0	4
41	M	RIS 8mg	13	4

Abbreviations: BZD, benzodiazepine dose expressed as diazepam equivalent dose; Anticholin, anticholinergic drugs expressed as biperiden equivalent dose; HPD, haloperidol; SPD, sulpiride; LP, levomepromadine; BPD: bromperidol; CP, chlorpromadine; ZTP, zotepin.

system of the brain. Findings from postmortem studies of GABAA receptor binding have been inconsistent, and have included reports of an increase (Hanada et al., 1987; Benes et al., 1992), a decrease (Squires et al., 1993), and no change (Pandey et al., 1997). Studies that have used SPECT (Busatto et al., 1997; Verhoeff et al., 1999; Abi-Dargham et al., 1999) or PET (Asai et al., 2008) to measure GABA receptor binding have reported that it is unchanged. GAD is the key enzyme in GABA synthesis. Postmortem studies of schizophrenia patients have reported that GAD levels are decreased in the nucleus accumbuns, amygdala, hippocampus, and putamen (Bird et al., 1977), and unchanged in the ACC (Benes et al., 2000). It has been shown that GAD65 and GAD67 mRNA are increased in the dorsolateral PFC and in the occipital cortex of schizophrenia patients (Dracheva et al., 2004). A reduction in GAD67 mRNA-containing neurons in the ACC has been reported in schizophrenia patients (Woo et al., 2004).

Table 4
The ratio of gray matter and white matter in each ROI.

		Schizophrenia		Control	p
		TYP	ATY		
GM in ACC	(all)	0.38 ± 0.08		0.37 ± 0.05	n.s.
	(all)	0.39 ± 0.08	0.37 ± 0.07		n.s.
WM in ACC	(all)	0.22 ± 0.07		0.22 ± 0.07	n.s.
	(all)	0.22 ± 0.07	0.21 ± 0.08		n.s.
GM in ltBG	(rt handed)	0.34 ± 0.11		0.35 ± 0.10	n.s.
	(rt handed)	0.37 ± 0.13	0.32 ± 0.08		n.s.
WM in ltBG	(rt handed)	0.62 ± 0.12		0.63 ± 0.11	n.s.
	(rt handed)	0.59 ± 0.13	0.65 ± 0.10		n.s.

Abbreviations: GM, gray matter; WM, white matter; rt handed, right handed; ACC, anterior cingulate cortex; ltBG, left basal ganglia; TYP, the patients taking typical antipsychotics with or without concomitant atypical antipsychotics; ATY, the patients taking atypical antipsychotics without concomitant typical antipsychotics; n.s., not significantly different in two sample *t*-test.

Our study has demonstrated that the in vivo GABA concentrations of the ACC and the ltBG did not differ significantly between medicated chronic schizophrenia patients and healthy control subjects.

4.3. Higher ltBG GABA concentration in the TYP group than the ATY group

Animal studies have reported that typical antipsychotics increased the expression of GAD67 and GAD67 mRNA in the basal ganglia (Chen and Weiss, 1993; Johnson et al., 1994; Jolkkonen et al., 1994; Delfs et al., 1995a,b; Laprade and Soghomonian, 1995; Sakai et al., 2001), but that administration of atypical antipsychotic drugs did not (Delfs et al., 1995a; Sakai et al., 2001). Our observation of significantly higher GABA concentrations in the TYP group compared to the ATY group is therefore compatible with the findings of these animal studies. This difference in GABA concentration may be reflected in the dyskinetic effects of typical antipsychotics (Delfs et al., 1995b; Sakai et al., 2001), although the severity of dyskinesia did not differ significantly between the two groups of patients in the present study (data not shown). The true extent of the influence of typical and atypical antipsychotics on GABA concentration in the present patient sample remains unknown since we did not include unmedicated patients. Unmedicated schizophrenia patients might be expected to have lower GABA concentrations than patients treated with typical antipsychotics, but further studies would be necessary to test this hypothesis.

4.4. Relationship between GABA concentration and the dose of antipsychotics in the ACC, and the dose of anticholinergics in the ltBG

Results from animal studies have suggested that antipsychotic medication may influence the GABA system in the cingulate cortex (Sharp et al., 1994, 2001; Vincent et al., 1994; Squires and Saederup, 2000). Zink et al. (2004) reported that both clozapine and haloperidol increased $GABA_A$ receptor binding in the ACC, while only haloperidol increased $GABA_A$ receptor binding in the basal ganglia. These animal studies indicated that both atypical and typical antipsychotics have an effect on the GABA system in the cingulate cortex.

Previous MRS studies have examined the effect of antipsychotic treatment on the combined signals of glutamate, glutamine and GABA (Glx). Although one study reported a decrease in Glx levels following antipsychotic treatment (Choe et al., 1996), another reported no significant difference in the PFC following treatment with risperidone (Szulc et al., 2005). A further study reported no change in the cingulate cortex following a switch from typical antipsychotics to olanzapine (Goff et al., 2002). It is difficult to estimate the exact effect of antipsychotics on GABA concentrations from these studies. In a previous study, we found no significant correlation between glutamate or glutamine concentrations and the dose of antipsychotics (Tayoshi et al., 2009).

The present study found a significant negative correlation between the GABA concentration in the ACC and the dose of antipsychotics i.e. a higher dose of antipsychotics was associated with a lower concentration of GABA in the ACC.

All antipsychotic medications, including both typical and atypical antipsychotics, may decrease the GABA concentration in the ACC. Although no difference was found in the GABA concentration in the ACC of medicated patients compared to healthy control subjects, it would be reasonable to hypothesize that unmedicated patients might show a higher GABA concentration in the ACC. Studies of unmedicated patients would provide valuable insights into this issue.

The significant positive correlation found between the dose of anticholinergic medication and the GABA concentration in the ltBG may be of clinical significance. The striatum receives dense cholinergic innervations from local interneurons, and the main synaptic targets of these cholinergic interneurons are GABAergic projection neurons (Graybiel, 1990; Izzo and Bolam, 1988). Muscarinic receptors on the GABA neurons are known to reduce GABA-mediated synaptic potentials and GABA release (Calabresi et al., 2000). It has been proposed that blockade of muscarinic stimulation by anticholinergic medication may increase the GABA concentration in the basal ganglia of schizophrenia patients.

4.5. Limitations

The present study has certain limitations. Firstly, the volume of the ROIs was set relatively high in order to measure the GABA concentrations. The volume was 27 ml, and the ROIs might therefore have contained heterogeneous tissues. Different results might have been obtained if the GABA concentration had been measured with smaller ROIs containing more homogenous tissues. Secondly, schizophrenia is thought to be a heterogeneous disorder, and the inherent heterogeneity of the schizophrenia patients might have affected the results, although the fact that most of the schizophrenia patients in our study were diagnosed as paranoid type may have minimized this effect. However, had the schizophrenia patients been divided into subgroups on the basis of other factors (e.g., genetic factors), significant differences in the GABA concentration between some of the schizophrenia subgroups and healthy control subjects might have been revealed. Thirdly, since antipsychotic medication may influence GABA concentrations, measurements in patients not taking antipsychotic medication would have provided valuable insights. Finally, although around half of the participants in the present study were female, we did not control for the effect of the menstrual cycle, a factor which may have an effect on GABA concentrations (Epperson et al., 2005).

5. Conclusion

Using ^{1}H-MRS, we have been the first to measure GABA concentrations in schizophrenia patients, but have found no differences in either the ACC or the ltBG between patients and healthy control subjects. Among the schizophrenia patients, the GABA concentration in the ltBG was higher in those taking typical antipsychotics than in those taking atypical antipsychotics. The GABA concentration in the ACC was found to be negatively correlated with the dose of the antipsychotics. Although the underlying mechanism is unclear, our results suggest that antipsychotic medication may induce changes in GABA concentration, and that these changes are dependent upon the type of medication. Antipsychotic medication may conceal inherent differences in GABA concentrations between schizophrenia patients and healthy controls.

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The funding sources had no involvement in the study design; collection, analysis and interpretation of the data; or writing of the report and the decision to submit it for publication.

Contributors

Author Tayoshi designed the study, wrote the protocol, managed the literature search, and undertook the statistical analysis. Author Nakataki recruited study participants and undertook additional statistical analysis. Authors Sumitani, Taniguchi, Shibuya-Tayoshi, Numata, and Iga recruited study participants. Author Harada operated the ^{1}H-MRS. Author Ueno also managed the literature search and recruited study participants. Author Ohmori managed the progress of the entire study.

Conflict of interest

The authors have no conflict of interest to declare.

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