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FKBP5, *SERT* and *COMT* mRNA expressions in the peripheral leukocytes during menstruation cycle in healthy reproductive females

Sawako Kinouchi [a], Jun-Ichi Iga [a], Shu-Ichi Ueno [a,b,*], Ken Yamauchi [a], Shusuke Numata [a], Hongwei Song [a], Satsuki Sumitani [a], Sumiko Shibuya-Tayoshi [a], Mari Haku [c], Toshiyuki Yasui [d], Minoru Irahara [d], Kyoko Morita [e], Kazuhito Rokutan [e], Tetsuro Ohmori [a]

[a] *Department of Psychiatry, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan*
[b] *Department of Community and Psychiatric Nursing, School of Health Sciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8509, Japan*
[c] *Department of Maternal and Pediatric Nursing, School of Health Sciences, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima, 770-8509, Japan*
[d] *Department of Obstetrics and Gynecology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan*
[e] *Department of Stress Science, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan*

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Abstract

There have been several evidences that the mRNA expressions in the peripheral leukocytes may indicate not only physical but also psychological states. The purpose of this study is whether the mRNA expressional changes in the leukocytes are related to the mental states across the menstrual cycle in reproductive healthy female subjects. Thirty-eight female subjects (22.4 ± 1.4 year-old) were participated in this study at three menstruation cycle periods (menstrual, follicular and luteal phase). The *FKBP5* (FK506-binding protein gene), *SERT* (serotonin transporter gene) and *COMT* (catechol-*o*-methyltransferase gene) mRNA expressions in the leukocytes were determined with hormonal data. The psychological changes were assessed with self-rating hospital anxiety and depression scale (HADS). Only one thirds of subjects ($n = 12$) had regular menstrual cycles during the experiment. So we analyzed the data from these 12 subjects. The anxiety score of each subject was changed across the menstrual cycle (Friedman test: $P < 0.05$). The *FKBP5* mRNA expression was significantly lower in the follicular phase than in the other phases but no changes were seen in either *SERT* or *COMT* mRNA expressions among the phases. In conclusion, there are differences of HADS anxiety score and *FKBP5* mRNA expression in the leukocytes across the menstrual cycle but there is no correlation between anxiety scores and FKBP5 mRNA.

Keywords: Menstrual cycle; HADS; mRNA expression; Leukocytes; Real time-PCR method

Approximately 80% of reproductive women experience physical and psychological symptoms during menstrual period [13]. The cases with sevère symptoms seen only in the late luteal phase are diagnosed as premenstrual syndrome (PMS) and a psychiatric disorder related to PMS is called premenstrual dysphoric disorder (PMDD), which are prevalent disorders among women of reproductive age [10]. According to the DSM-IV criteria [1,2], 3–8% of women meet the strict criteria for PMDD [10]. Those disorders have multi-dimensional symptoms and may include diverse physiologic systems although their etiology is still unknown.

Previous studies including our own have been reported that altered mRNA expressions in the peripheral leukocytes might indicate the pathophysiology of the neuronal changes in mental disorders [14,15,18,19,21]. For example, the serotonin transporter gene mRNA expression in the leukocytes of depressive

* Corresponding author at: Department of Community and Psychiatric Nursing, School of Health Sciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8509, Japan. Tel.: +81 88 633 9023; fax: +81 88 633 9023.
E-mail address: shuichi@medsci.tokushima-u.ac.jp (S. Ueno).

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patients is higher than that in healthy control subjects [14]. So it is useful to study the changes of the peripheral leukocytes not only in the immune and blood diseases but also in the central nervous system disorders.

Before studying the menstruation-related disorder patients, we tried to measure the changes with mental status across the menstruation cycle in healthy female subjects. Both biological and psychological changes across the menstrual cycle were studied simultaneously. We selected three gene expressions in the peripheral leukocytes as biological markers: *FKBP5* (FK506-binding protein; the FKBP51 gene which may be induced by glucocorticoids and its polymorphism is reported to be associated with antidepressant effects [6], *SERT* (5HTT; SLC6A4, the serotonin transporter gene) which codes a target protein of antidepressants used in PMS or PMDD treatment [9], *COMT* (catechol-*o*-methyltransferase gene) which codes COMT protein that degrades catecholamines and its soluble isoform is reported to be inhibited transcriptionally by estrogen [16,26]. The mRNAs of three genes are expressed in the leukocytes as suggested by the expression profile of analysis of EST counts in UNIGENE in NCBI home page (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer) or our previous study [14], and quantitationally measured by real time-PCR method. For assessing the psychological state during menstruation, we used a self-rating scale, hospital anxiety and depression scale (HADS) score [27].

Thirty-eight female volunteers (mean age: 22.4 ± 1.4 year-old) were recruited from the university students of midwifery course. All subjects were in good health without a history of either psychiatric or serious somatic diseases. They were un-medicated during this study. Complete blood cell counts, liver function and hormone levels (LH: luteinizing hormone; FSH: follicle-stimulating hormone; TSH: thyroid-stimulating hormone; PRL: prolactin, free T3, free T4; E1: estrone; E2: estradiol and progesterone) were measured (SRL, Tokyo, Japan) before this study and there were no abnormal findings. They signed written informed consent approved by the Ethical Committee of The University of Tokushima School of Medicine.

Basal body temperature with menstrual memory scale was measured by each subject every morning. They recorded the period of menstruation and premenstrual symptoms in the table if there were. The dates of psychological assessment and blood drawing in each phase of menstrual cycle were determined by themselves. Before starting this study, the subjects checked their own menstruation cycles for about 6 months at least. The day in the menstruation phase was 2.7 ± 0.5 days after the onset of menstruation and the day in follicular phase was 9.8 ± 1.8 days after the onset of menstruation. In luteal phase, the day chosen was eighth day after the body temperature raised high phase period (6.8 ± 3.4 days prior to onset of menstruation).

Hospital anxiety and depression scale (HADS) [27], which consists of seven questions about anxiety and depression each, were performed to evaluate anxiety and depressive state in the menstrual phase. The score of each question ranges from 0 to 3 (higher score shows more serious symptom). Cut off point is eight in both anxiety and depression scales [7].

For the hormone assays and the quantitation of the mRNAs in the leukocytes, the subjects underwent venous blood drawing in the morning of the same day they performed self-rating HADS scores in each phase.

The blood samples were placed in the refrigerator just after sampling and the maximum time between taking the sample and centrifugation was 6 h. LH, FSH, PRL, E1, E2 and progesterone were determined each phase by ELISA by a third party (SRL, Tokyo, Japan).

The expressional profiles of the mRNA levels are determined with quantitative real time-PCR method [14]. Total RNA was extracted from the leukocytes of whole blood samples using PAXgene Blood RNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Residual genomic DNA was digested with RNase-free DNase I. One to five micrograms of total RNA was used for cDNA synthesis by oligo (dT) primers and Powerscript Reverse Transcriptase (BD Biosciences, Tokyo, Japan). Primer and probe sequences were selected at exon-intron boundary of *FKBP5*, *SERT* and *COMT* and optimized melting temperatures (Nihon Gene Research Lab's Inc., Sendai, Japan). Primers and Hybridization probes were as followed: 5′-AGAAAGTGCTGGAAGTA-3′ and 5′-CCTTTTTCATTAGTGACC-3′ primers; 5′-TTCAAGAAGTT-TGCAGAGCAGGATGCCA-3′Fluorescein and 5′-LCRed640-GGAAGAGGCCAATAAAGCAATGGGCAAG-3′ for *FKBP5*; 5′-TCTATGGCATCACTCAGTT-3′ and 5′-TGGAAAAGTCG-TAGTTGTG-3′-primers, 5′-AACAGGAGAAACAGAGGGC-TGATGGC-3′Fluorescein and 5′-LCRed640-ACCCAGCAGA-TCCTCCAGAACCACC-3′ for *SERT*; 5′-TCGGCTGGAAC-GAGTTCA-3′ and 5′-CGTCCACGATCTTGCCTT-3′ primers, 5′-GGTTCAGGATGCGCTGCTCCTTGGT-Fluorescein and 5′-LCRed640-TCACCCATGAGCAGGTTGTGGATGG-3′ for *COMT*. Quantitative real time-PCR was performed with Light-Cycler (Roche Diagnostics, Tokyo, Japan). Two housekeeping genes were used for normalization (glucose-6-phosphate dehydrogenase; *G6PD* and hypoxanthine guanine phosphoribosyltransferase; *HPRT*, Qiagen, Tokyo, Japan). There was no expressional difference in either house keeping gene, so we used *G6PD* as an internal standard. Measurement of each gene expression was held in triplicates. Amplification of the product in the real time-PCR method was confirmed by agarose gel electrophoresis.

Statistical calculations were carried out using the SPSS Statistical Software Package 11.5 (Tokyo, Japan). Menstrual cycle effects on HADS score in each subject were analyzed by Friedman test. The changes during menstruation cycles were analyzed by two-way analysis of variance (ANOVA) ("three menstruation cycles"×" subjects") with repeated measures followed by Bonferroni post hoc test. P values less than 0.05 were considered to be statistically significant in all tests. Data are presented as mean ± standard deviation.

All subjects showed no abnormal findings in laboratory examination. A gynecologist (T.K.) assessed the phase of menstrual cycles from the graphical data of basal body temperature and gonadal steroid levels. The critical decision points were body temperature (the length of high-temperature period), the data of E2 and progesterone levels. Twenty four subjects were diag-

Table 1
Endocrine examination in each menstrual phase

	Menstruation	Follicular	Luteal
LH (mIU/ml)	2.70 ± 1.78	5.69 ± 2.74	2.63 ± 3.22
FSH (mIU/ml)	4.81 ± 1.78	5.57 ± 1.41	2.15 ± 1.10
PRL (ng/ml)	14.2 ± 12.1	12.1 ± 10.1	14.4 ± 11.6
E1 (pg/ml)	25.6 ± 12.8	44.4 ± 18.5	75.8 ± 24.0
E2 (pg/ml)	28.3 ± 10.4	48.8 ± 18.2	131.2 ± 52.1
Progesterone (ng/ml)	0.66 ± 0.23	0.63 ± 0.26	9.54 ± 2.47

mean ± S.D. $n = 12$.

Table 2
Hospital anxiety and depression scale

	Menstruation	Follicular	Luteal
HADS-A	4.3 ± 3.2 (11)	3.8 ± 4.7	4.8 ± 3.5
HADS-D	5.3 ± 2.5	4.2 ± 3.0	5.3 ± 3.1 (11)
HADS-total	9.6 ± 5.2 (11)	8.0 ± 6.6	10.3 ± 4.9 (11)

The cut-off point is eight in anxiety and depression score. mean ± S.D. $n = 12$ (parenthesis: numbers tested) There was a significant difference among control subjects in HADS-A (Friedman test: $P < 0.05$).

nosed as luteal insufficiency or anovulatory cycle in that period. Two subjects showed amenorrhea during this study. They were excluded from data analyses. Finally, we analyzed 12 subjects with regular menstrual cycles and they did not meet criteria for PMDD according to DSM-IV [1]. The values of LH, FSH, PRL, E1, E2 and progesterone across the menstruation cycle were shown in Table 1.

The scores of HADS-A score (anxiety), HADS-D score (depression) and HADS-total score in each phase were shown in Table 2. When menstrual cycle effects on HADS score in each subject were analyzed by Friedman test, only HADS-A score significantly changed among the phases ($P = 0.048$). Comparison of HADS-A, HADS-D or total HADS scores using two-way ANOVA showed no significant differences across the menstrual cycle.

Fig. 1 shows mRNA expression of *FKBP5*, *SERT* and *COMT* in three phases calculated as a percentage of the mean values in each subject. The post hoc analysis by Bonferroni test revealed that the FKBP5 mRNA expression was significantly lower in the follicular phase than those in either menstruation or luteal phase ($P = 0.040$ and 0.029, respectively). But there was no significant difference in *SERT* or *COMT* mRNA levels among the phases. We could not find any correlation between three HADS scores and these three gene expressions.

Although 38 women participated in this study, 26 subjects showed luteal insufficiency or anovulatory cycle or amenorrhea. Then, we utilized only 12 subjects (about one thirds of participants) who were appropriate for this study. A study reported that 72% of 19-year-old women and 67% of 24-year-old women experienced problem with menstruation [22] but it was unexpected that more than two thirds of subjects have menstrual disturbances in that period. This fact may be due to their youth (22.0 ± 1.2 year-old) or their irregular daily schedule even in healthy controls without any symptoms or signs. This shows that the exact assessment of menstrual cycles and the appropriate selection of subjects are important for studies across the menstrual cycle.

We chose self- rating questionnaire, HADS, for the assessment of psychological changes in these three menstruation phases. HADS is a short but useful self-rating test that measures both anxiety and depressive score simultaneously. Bjelland et al reviewed that HADS is a suitable tool in assessing anxiety and depression in the general population [7]. We found significant fluctuation of anxiety over the menstrual cycles. It is expected that HADS is also useful for evaluating the psychological changes in the PMS or PMDD patients.

For the purpose of studying the mRNA changes across the menstruation cycle, we select *FKBP5* as a hormonal marker. Because Glucocorticoid induces *FKBP5* mRNA expression [4] and its response may be a suitable marker to assess individual glucocorticoid sensitivity [25]. Single-nucleotide polymorphisms in FKBP5 were reported to associate with response to antidepressants, the recurrence of depressive episodes and increased intracellular FKBP51 protein [6]. It is interesting that the *FKBP5* mRNA expression was significantly lower only in the follicular phase in this study. Kester et al. demonstrated that progestin regulates FKBP51 [17] and overexpression of *FKBP5* might attenuate progestin responsiveness [12]. It can be postulated that the increased progesterone level may induce *FKBP5* mRNA in the luteal phase. In the menstrual phase, the *FKBP5* expression was also high compared to the follicular phase. It is suggested that even the level of progesterone in the serum is low in that phase but that its effect continues in several days after the menstruation started because the maximum induction of *FKBP5* mRNA with progestin R5020 remained elevated for at least 24 h

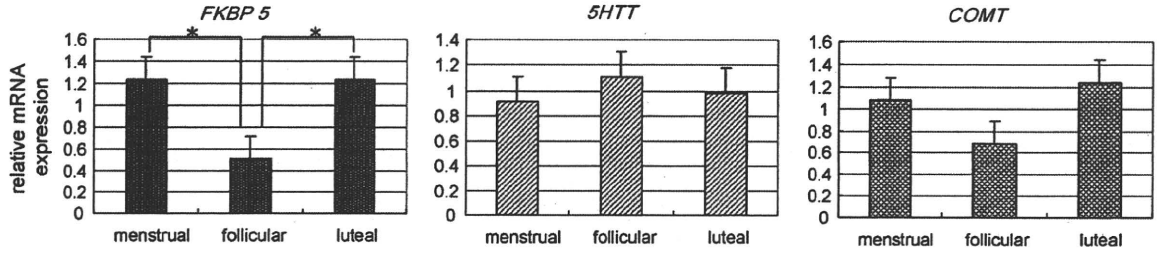

Fig. 1. Effects of each menstrual cycle on mRNA expression of *FKBP5*, *SERT* and *COMT* in the peripheral leukocytes. Relative mRNA expression of three genes was shown in the vertical axis (the bars indicate S.D.). *FKBP5* mRNA expression was significantly lower in the follicular phase compared with the other phases (two-way ANOVA and Bonferroni test: menstruation vs. follicular; $P = 0.040$, follicular vs. luteal; $P = 0.029$). There was no significant difference in 5-HTT or COMT mRNA levels during the phases.

after treatment in vitro [12]. Another possibility is that the other factors may be related to its elevation, for example, glucocorticoid level. The concentration of progesterone is reported to be not related to PMS directly [11] and the relation between the elevation of the *FKBP5* mRNA and the psychological symptoms are not known yet.

On the other hand, serotonergic system also plays an important role in the pathogenesis of premenstrual symptoms because selective serotonin reuptake inhibitors (SSRIs) are effective in the treatment of PMS and PMDD [8,9]. Previous studies have indicated that whole blood serotonin levels [20], platelet uptake and content of serotonin in the luteal phase are lower in patients with PMS compared to control [3]. We have reported that *SERT* mRNA is expressed in the peripheral leukocytes and that its expression in depressive state is higher than that in control subjects and decreased after SSRI treatment [14]. In this study, we found no significant changes of *SERT* mRNA level in the leukocytes across the menstrual cycle in healthy female subjects. This result suggests that serotonergic system may not be involved primarily in the mood change during menstruation cycles. Although *SERT* mRNA in the leukocytes was not changed transcriptionally in normal females, there is a possibility that SERT expression may be posttranscriptionally modified and that the expression of *SERT* mRNA in the brain may be affected during menstrual cycles.

We also measured *COMT* mRNA expression levels in the leukocytes. Several studies have shown that *COMT* mRNA expression in the frontal cortex is related to the level of cognitive function [5,24]. Total *COMT* mRNA expression in the leukocyte was not changed during the menstrual cycles. However, we analyzed the mRNA expressions of *COMT* soluble and membrane-bound type together. Further studies to analyze each type of *COMT* mRNA are required because estrogen is reported to modify soluble type of *COMT* transcript while the membrane-bound type of *COMT* mRNA expression is higher in the brain [23].

In conclusion, our study suggests that the *FKBP5* mRNA but not either *SERT* or *COMT* mRNA expression changes across the menstrual cycle in the peripheral leucocytes of healthy female subjects. They showed a fluctuation of anxiety across the menstruation cycle but there were no correlation between anxiety and *FKBP5* mRNA expression. Since the sample sizes were small and this study was done only with healthy controls, further studies to analyze the psychological and biological effect of the menstrual cycle in patients with menstruation-related disorders are required.

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Subjective and objective quality of life, levels of life skills, and their clinical determinants in outpatients with schizophrenia

Hirofumi Aki *, Masahito Tomotake, Yasuhiro Kaneda, Jun-ichi Iga, Sawako Kinouchi, Sumiko Shibuya-Tayoshi, Shin-Ya Tayoshi, Ikuyo Motoki, Kazuhiko Moriguchi, Satsuki Sumitani, Ken Yamauchi, Takahide Taniguchi, Yasuhito Ishimoto, Shu-ichi Ueno, Tetsuro Ohmori

Department of Psychiatry, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima, Tokushima 770-8503, Japan

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Abstract

The purpose of the present study is to investigate the relationships among subjective and objective quality of life (QOL), and levels of life skills, and their clinical determinants in outpatients with schizophrenia by using schizophrenia disease-specific QOL measures. Data collected from 64 outpatients were analyzed. Subjective QOL was measured with the Schizophrenia Quality of Life Scale (SQLS) and objective QOL with the Quality of Life Scale (QLS). Patients' family members completed the Life Skills Profile (LSP). Clinical symptoms were also assessed with several scales including the Brief Psychiatric Rating Scale (BPRS) and the Calgary Depression Scale for Schizophrenia (CDSS). Only the motivation/energy scale, but not the other scales of the SQLS, correlated with the QLS. The LSP rated by the family showed significant correlations with both the SQLS and the QLS. The CDSS score predicted each scale of the SQLS, and the BPRS negative symptoms score predicted the QLS. The LSP was predicted by the BPRS negative symptoms score and the CDSS score independently. These results indicate that the patient's QOL could be predicted by the life skills measured by a family member and suggest that active treatment for depressive and negative symptoms might be recommended to improve the patient's QOL and life skills.

Keywords: Quality of life; Life skill; Schizophrenia

1. Introduction

Although there seems to be no unanimous definition of quality of life (QOL), QOL is generally thought to include life satisfaction, social functioning, daily living activities, and physical health, and it has been recognized as an important indicator of how well patients with schizophrenia can function (Lehman, 1998; Meltzer, 1992, 1999). QOL has been measured from two different viewpoints. One is subjective QOL rated by patients themselves, and another is objective QOL rated by observers. Although patients with schizophrenia were thought to be unable to assess their QOL by themselves because of their cognitive deficit function, it would be reasonable to assume that symptomatically

* Corresponding author. Tel.: +81 88 633 7130; fax: +81 88 633 7131.
E-mail address: yxpwc180@ybb.ne.jp (H. Aki).

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stabilized patients are able to evaluate their QOL by themselves (Voruganti et al., 1998). To date, several assessment scales have been developed to assess subjective and objective QOL (Heinrichs et al., 1984; Wilkinson et al., 2000). However, the relationship between these two QOL perspectives is not clear. Fitzgerald et al. (2001) reported that some significant correlations were found between subjective and objective QOL measures in schizophrenia.

The clinical factors related to levels of QOL have been variously reported. Several studies have suggested depressive mood may be the most important determinant for subjective QOL (Dickerson et al., 1998; Fitzgerald et al., 2001; Huppert et al., 2001; Reine et al., 2003). Of these studies, only Reine et al. used the Calgary Depression Scale for Schizophrenia (CDSS), which specifically measures depressive rather than positive or negative symptoms or antipsychotic-induced side effects (Rocca et al., 2005). Other studies reported that positive symptoms (Norman et al., 2000) or akathisia symptoms as well as the total severity of psychopathology (Awad et al., 1997) predicted subjective QOL. In some studies, the severity of negative symptoms (Fitzgerald et al., 2001; Strejilevich et al., 2005) or the presence of tardive dyskinesia (Browne et al., 1996) was reported to be associated with a poor objective QOL. Levels of insight into the illness showed no significant relationship with QOL levels (Browne et al., 1998). In addition to clinical symptoms, socio-demographic factors also influence objective QOL of patients with schizophrenia (Caron et al., 2005). The variance in previous findings might derive from the difference of the QOL measures used and the difference of the subjects investigated. Further research is needed to clarify clinical factors influencing subjective and objective QOL using appropriate measures.

Another approach to measuring QOL of patients with schizophrenia is to use the assessment by family members of patients. We used the Life Skills Profile (LSP) for this purpose. The LSP developed by Rosen et al. (1989) is a suitable measure of function and disability associated with schizophrenia. The LSP can be used by family members of patients as well as by community housing managers or professional staff (Rosen et al., 1989; Parker et al., 1991), and it shows good internal consistencies and validity (Trauer et al., 1995). Up to now, there have been few reports concerning the influence of psychiatric symptoms or of, the dosage and side effects of drugs, on the scores of the LSP.

In this study, we investigated relationships among patient-rated subjective QOL, observer-rated objective QOL, and family-rated LSP in patients with schizophrenia. This study is the first trial to utilize patient-rated, observer-rated, and family-rated measures simultaneously. We also investigated their clinical determinants with multivariate analysis.

2. Material and methods

2.1. Subjects

Clinical data were collected at the Department of Psychiatry, Tokushima University Hospital, from April 26 to June 18, 2004. After getting written consent from all subjects, we selected 105 outpatients whose diagnoses had been confirmed by at least two psychiatrists according to the DSM-IV.

Subjects were excluded if they presented with any organic central nervous system disorder, epilepsy, mental retardation, severe somatic disorder, drug dependence, or alcohol dependence. We also asked their family members to complete the LSP. Of 105 family members, 64 gave us written consent and completed the questionnaire. Of 64, 46 were their parents, 6 their spouses, 6 their siblings, and 5 their children. Only one did not specify the relationship to the patient. Data from 64 family members and 64 corresponding outpatients were used for the statistical analysis.

The subjects in the present study were all stabilized and had been able to receive outpatient treatment regularly. Fifty had never been hospitalized during the previous 1 year, including 13 who had never had inpatient treatment, while 14 had inpatient treatment during the previous 1 year. Sixty-one subjects had followed the same antipsychotic regimen for at least 6 months before recruitment, while three subjects had slight changes in regimen during the previous 6 months; however, the three were judged as clinically stabilized by the treating psychiatrists.

2.2. Procedure

To assess subjective QOL, we used the Schizophrenia Quality of Life Scale (SQLS) (Wilkinson et al., 2000; Kaneda et al., 2002). Objective QOL was evaluated using the Quality of Life Scale (QLS) (Heinrichs et al., 1984, 2001). Psychotic symptoms, including positive and negative symptoms, were evaluated using the Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham, 1962; Miyata et al., 1995). Depressive symptoms were assessed using the CDSS (Addington et al., 1993; Kaneda et al., 2000). Drug-induced extrapyramidal symptoms were assessed using the Drug-Induced Extrapyramidal Symptoms Scale (DIEPSS) (Inada, 1996). Life skills were assessed by family members living with the subjects using the LSP (Rosen et al., 1989; Hasegawa and Ogawa, 1997).

The SQLS is a self-reported, 30-item questionnaire for measuring QOL specific to patients with schizophrenia with good reliability and validity (Wilkinson et al., 2000; Kaneda et al., 2002). It is composed of three scales:

psychosocial, motivation/energy and symptoms/side effects. Lower scores indicate higher levels of subjective QOL.

The QLS is a measure to assess objective QOL by means of a semistructured interview. The reliability and validity of the scale have been verified (Heinrichs et al., 1984, 2001). The ratings are based upon patients' self-report and observers' judgment about the patients' functioning and life circumstances. This instrument has the following four subscales: interpersonal relations, instrumental role, intrapsychic foundation, and common objects and activities. Higher scores indicate higher levels of objective QOL. Some of the authors, who were all experienced psychiatrists, conducted the interviews according to the Evaluation Manual for the QLS (Heinrichs et al., 2001).

According to the previous study, the sum of the scores for four symptoms (suspiciousness, hallucinatory behavior, conceptual disorganization, and unusual thought content) of the BPRS was considered to be a positive symptom score (McCreadie et al., 1990; Arango et al., 2003; Josiassen et al., 2005), and the sum of the scores for four other symptoms (emotional withdrawal, motor retardation, blunted affect, and disorientation) of the scale was considered to be a negative symptom score (Meltzer et al., 1990; Poulin et al., 2003; Kaneda and Ohmori, 2005).

The CDSS was specifically developed to distinguish depressive symptoms from positive or negative symptoms or antipsychotic-induced side effects in schizophrenia. A higher score indicates a greater level of depression. The reliability and validity of the scale have been verified (Addington et al., 1993; Kaneda et al., 2000).

The DIEPSS is composed of nine items using a 5-point scale that ranges from 0 to 4. In this study, we used the rating of overall severity, which is one of the items of the DIEPSS. The reliability and validity of the scale have been verified (Inada, 1996).

Life skills were assessed using the LSP. The LSP was designed by Rosen et al. (1989) to assess survival and adaptation in the community by individuals with severe mental illness (Norman et al., 2000). The reliability and validity of the scale have been verified (Rosen et al., 1989; Hasegawa and Ogawa, 1997). The LSP has the following five subscales: self-care, non-turbulence, socialization, communication, and responsibility.

All the scales except for the SQLS and the LSP were carried out by the authors, all of whom were experienced psychiatrists. Inter-rater consistencies of the CDSS and the BPRS in our group have been shown to be satisfactory (Kaneda et al., 2000; Numata et al., 2006).

2.3. Statistical analysis

Pearson's correlation coefficients were calculated to study the relationships among the SQLS, the QLS and the LSP. Statistical significance was adjusted for multiple comparisons (Bonferroni correction). The SQLS scale score, the QLS total score and the LSP total score were chosen as dependent variables. Stepwise regression analyses were done to assess the independent contribution of other clinical variables (duration of illness, number of hospitalization, dose of antipsychotic medication, the BPRS positive symptoms score, the BPRS negative symptoms score, the DIEPSS score, and the CDSS score) to each dependent variable. Statistical analyses were done with the Statistical Package for the Social Sciences, version 11.5J. A P-value <0.05 was taken to indicate statistical significance.

3. Results

Demographic characteristics and means and standard deviations of the clinical indices are presented in Table 1.

Table 1
Demographic characteristics of subjects

N (M/W)		64(29/35)
Age (years)		41.8(12.7)
Duration of illness (years)		12.5(9.2)
Number of hospitalizations		1.4(1.5)
Dose of antipsychotics (mg/day)*		535.4(463.5)
Type of schizophrenia (n)	Residual	8
	Paranoid	49
	Catatonic	1
	Disorganized	6
Marital state (n)	Married	10
	Never married	48
	Divorced	5
	Widowed	1
Social state (n)	Full time	7
	Part time	5
	No employment	52
BPRS	Total	31.7(8.4)
	Positive	8.3 (3.8)
	Negative	9.1(3.3)
DIEPSS (Overall)		0.4(0.7)
CDSS (Total)		2.8(3.4)
SQLS	Psychosocial	39.9(18.7)
	Motivation/energy	49.3(19.9)
	Symptoms/side effects	25.5(17.7)
QLS (Total)		64.8(29.7)
LSP (Total)		133.7(13.1)

Results are means with S.D.; *Chlorpromazine equivalent; BPRS, Brief Psychiatric Rating Scale; DIEPSS, Drug-Induced Extrapyramidal Symptoms Scale; CDSS, Calgary Depression Scale for Schizophrenia; SQLS, Schizophrenia Quality of Life Scale; QLS, Quality of Life Scale; LSP, Life Skills Profile.

Table 2
Correlation coefficients among SQLS, QLS and LSP

	SQLS			QLS				
	Psychosocial	Motivation/ energy	Symptoms/side effects	Total	Interpersonal relations	Instrumental role	Intrapsychic foundation	Common objects and activities
QLS								
Total	−0.29	−0.49**	−0.28					
Interpersonal relations	−0.22	−0.43**	−0.20					
Instrumental role	−0.37*	−0.47**	−0.32					
Intrapsychic foundation	−0.29	−0.45**	−0.26					
Common objects and activities	−0.17	−0.40*	−0.34					
LSP								
Total	−0.47**	−0.41*	−0.46**	0.55**	0.48**	0.56**	0.49**	0.47**
Self-care	−0.40*	−0.32	−0.43**	0.52**	0.46**	0.54**	0.45**	0.49**
Non-turbulence	−0.44*	−0.25	−0.43**	0.16	0.08	0.24	0.17	0.13
Socialization	−0.36	−0.44**	−0.28	0.63**	0.57**	0.57**	0.57**	0.50**
Communication	−0.33	−0.31	−0.37*	0.37	0.32	0.39*	0.33	0.27
Responsibility	−0.24	−0.17	−0.25	0.26	0.22	0.29	0.23	0.26

SQLS, Schizophrenia Quality of Life Scale; QLS, Quality of Life Scale; LSP, Life Skills Profile; *$P<0.05$, **$P<0.01$ (Bonferroni correction).

All subjects were Japanese, with 29 males and 35 females. The average age was 41.8 years (S.D.=12.7). Subtype diagnoses included 49 paranoid type, 8 residual type, 6 disorganized type, and 1 catatonic type. Ten of the subjects were married, 48 had never been married, five had been divorced, and one was widowed. We used the chlorpromazine conversion chart (Inagaki et al., 1998, 2001a,b,c) to determine the dosage of antipsychotic medication.

The correlations among the SQLS, the QLS and the LSP are shown in Table 2. The SQLS motivation/energy scale significantly and negatively correlated with the QLS total ($r=-0.49$, $P<0.01$), interpersonal relations subscale ($r=-0.43$, $P<0.01$), instrumental role subscale ($r=-0.47$, $P<0.01$), intrapsychic foundation subscale ($r=-0.45$, $P<0.01$), and common objects and activities subscale ($r=-0.40$, $P<0.05$). The SQLS psychosocial scale significantly and negatively correlated with the LSP total ($r=-0.47$, $P<0.01$), self-care subscale ($r=-0.40$, $P<0.05$), and non-turbulence subscale ($r=-0.44$, $P<0.05$). The SQLS symptoms/side effects scale also had significant and negative correlations with the LSP total ($r=-0.46$, $P<0.01$), self-care subscale ($r=-0.43$, $P<0.01$) and non-turbulence subscale ($r=-0.43$, $P<0.01$). The SQLS motivation/energy scale had significant and negative correlations with the LSP total ($r=-0.41$, $P<0.05$) and socialization subscale ($r=-0.44$, $P<0.01$). The LSP total score significantly and positively correlated with the QLS total ($r=0.55$, $P<0.01$), interpersonal relations ($r=0.48$, $P<0.01$), instrumental role ($r=0.56$, $P<0.01$), intrapsychic foundation ($r=0.49$, $P<0.01$), and common objects and activities ($r=0.47$, $P<0.01$). The LSP self-care subscale correlated significantly and positively with the QLS total ($r=0.52$, $P<0.01$), interpersonal relations ($r=0.46$, $P<0.01$), instrumental role ($r=0.54$, $P<0.01$), intrapsychic foundation ($r=0.45$, $P<0.01$), and common objects and activities ($r=0.49$, $P<0.01$). The LSP socialization subscale score also had significant and positive correlation with the QLS total ($r=0.63$, $P<0.01$), interpersonal relations ($r=0.57$, $P<0.01$), instrumental role ($r=0.57$, $P<0.01$), intrapsychic foundation ($r=0.57$, $P<0.01$), and common objects and activities ($r=0.50$, $P<0.01$).

Table 3
Results of multiple regression analysis on SQLS, QLS Total and LSP Total

Dependent variable	Independent variable	R^2	β*
SQLS			
Psychosocial	CDSS	0.405***	0.636***
Motivation/energy	CDSS	0.264***	0.514***
Symptoms/side effects	CDSS	0.197***	0.444***
QLS total	BPRS-negative	0.329***	−0.573***
LSP total	BPRS-negative	0.276***	−0.335**
	CDSS		−0.324**

SQLS, Schizophrenia Quality of Life Scale; QLS, Quality of Life Scale; LSP, Life Skills Profile; CDSS, Calgary Depression Scale for Schizophrenia; BPRS, Brief Psychiatric Rating Scale; *Standardized regression coefficient; **$P<0.01$, ***$P<0.001$.

Table 3 shows the results of stepwise regression analyses. The psychosocial scale score was significantly predicted only by the CDSS total score (β=0.636 P<0.001). The only significant predictor of the motivation/energy scale score was the CDSS total score (β=0.514 P<0.001). The symptoms/side effects scale score was also significantly predicted only by the CDSS total score (β=0.444 P<0.001). The QLS total score was significantly predicted only by the BPRS negative symptoms score (β=−0.573 P<0.001). The LSP total score was significantly and independently predicted by the BPRS negative symptoms score (β=−0.335 P<0.01) and the CDSS total score (β=−0.324 P<0.01).

4. Discussion

The primary goal of the present study was to examine how clinical factors influence patients' QOL and life skills. In addition, we investigated relationships among patient-rated subjective QOL, observer-rated objective QOL, and family-rated level of life skills. There were several important findings in this study. The results suggest what symptoms we should focus on in order to improve patients' QOL and life skills.

4.1. Relationship between subjective and objective QOL

Fitzgerald et al. (2001) reported some significant correlations between subscales of objective and subjective QOL measures in 174 outpatients. They used the QLS, a schizophrenia disease-specific objective QOL measure, and the self-report life satisfaction scale from the Schizophrenia Care and Assessment Program as a subjective QOL scale. We used a newly developed schizophrenia disease-specific subjective QOL measure, the SQLS, to investigate the relationship. It is of note that the motivation/energy score of the SQLS correlated with the QLS total and all subscales while the scores of the psychosocial scale of the SQLS correlated with only the instrumental role subscale but not other subscales of the QLS. The symptoms/side effects subscale of the SQLS did not significantly correlate with the total score or any subscale of the QLS. The motivation/energy subscale addresses various problems of motivation and activity, such as lacking the will to do things or engage in positive aspects of life (Wilkinson et al., 2000). It has been suggested that subjective assessment of motivation and activity might predict objective QOL. The absence of strong correlations between psychosocial or symptom/side effects scores of the SQLS and the QLS scores, however, suggests that these two QOL measures reflect different aspects of QOL.

4.2. Relationship between LSP and either subjective or objective QOL

Parker et al. (2002) reported no significant correlation between the LSP and subjective QOL. In contrast, we found the LSP total score, the LSP self-care subscale score, and the LSP non-turbulence subscale score correlated with psychosocial and symptoms/side effects scales of the SQLS. The LSP total score and socialization subscale score correlated with the motivation/energy scale. The LSP communication subscale score correlated with the symptoms/side effects score. However, the LSP responsibility subscale score did not correlate with any scale of the SQLS. The difference in results between the two studies may reflect the differences of subjects and QOL measures. Our subjects were composed of patients with schizophrenia, while their subjects included patients with schizophrenia, schizoaffective disorder, schizophreniform disorder, and bipolar disorder. We used the schizophrenia disease-specific subjective QOL measure, while they used the Quality of Life Index for Mental Health, which is not a measure specific to schizophrenia. Alternatively, the difference in the rater might contribute to the difference. The rater was the family member in our study but the community staff in theirs.

As for the relationship between objective QOL measure and the LSP, Norman et al. (2000) reported that the LSP assessed by psychiatrists was associated with objective QOL measured with the QLS. In agreement with their report, the LSP total, self-care and socialization subscales significantly correlated with the QLS total and the subscales. The LSP assessed by family members correlated with objective QOL. These results indicate that the LSP rated by the family is associated with both patient-rated subjective and observer-rated objective QOL in patients with schizophrenia. Assessment of patient life skill with the LSP by the family member may conveniently and accurately predict subjective and objective QOL.

4.3. The factors influencing subjective and objective QOL

Previous studies have found depressive symptoms predict subjective QOL (Dickerson et al., 1998; Fitzgerald et al., 2001; Sim et al., 2004). However, depressive symptoms in schizophrenia are difficult to distinguish from negative and drug-induced extrapyramidal symptoms (Addington et al., 1993). Recently, Reine et al. (2003) measured depressive symptoms with the CDSS, a scale specifically developed to measure depressive symptoms in schizophrenia, and replicated the previous finding. In the present study, using the CDSS and the SQLS, disease-specific measures of depressive

symptoms and subjective QOL, respectively, an association between depressive symptoms and subjective QOL was further confirmed. Objective QOL has been reported to be predicted by negative (Fitzgerald et al., 2001; Strejilevich et al., 2005) and extrapyramidal symptoms (Browne et al., 1996; Strejilevich et al., 2005). Our results also suggest negative symptoms predict objective QOL. Considering that the QLS was originally designed to evaluate deficit symptoms and the dysfunctions related to them (Heinrichs et al., 1984), the correlation between negative symptoms and the QLS scores seems to be reasonable. We did not find a significant correlation between objective QOL and extrapyramidal symptoms, probably because of relatively low levels of extrapyramidal symptoms in the present subjects. Positive symptoms have been reported to predict neither subjective (Dickerson et al., 1998; Fitzgerald et al., 2001; Sim et al., 2004) nor objective QOL (Fitzgerald et al., 2001; Browne et al., 1996; Strejilevich et al., 2005). Consistent with these previous studies, the present study suggests that positive symptoms do not predict subjective or objective QOL in stabilized outpatients with schizophrenia. In contrast, Norman et al. (2000) reported that positive symptoms were related to subjective QOL and that both positive and negative symptoms were related to objective QOL. Differences in the method and/or the subject population may account for the different findings.

4.4. The factors influencing the LSP

Parker et al. (2002) reported that the LSP correlated strongly with the Health of the Nation Outcome Scale, which assesses behavior (aggression, self-harm, substance abuse), impairment (memory, orientation, physical health), symptoms (mood disturbance, hallucinations, delusions), and social functioning (social relations, housing, activities). Their results suggest a good consistency between the two scales as measures of the functioning of patients with schizophrenia. Norman et al. (1999) reported that positive but not negative symptoms correlated with the LSP total and three subscales: social contact, communication, and self-care. They did not measure depressive symptoms. In contrast to their results, this study found that the BPRS negative symptom score and the CDSS total score predicted the LSP total independently, but positive symptoms did not predict it. One reason for the discrepancy may be different methods of analysis. We analyzed several clinical factors together using stepwise regression analyses, while they simply studied correlations between them. Another explanation may involve difference in the rater who completed the LSP. The patient's family members rated the LSP in our study while care managers did in their study. Although untrained, family members have a great advantage as the rater for the LSP because they know the patients' life skills thoroughly. Alternatively, the difference may be related to the difference in patient populations. Compared with their patients, our patients were older (41.8 vs. 30.9 years), with longer durations of illness (12.5 vs. 5.2 years) and with higher doses of antipsychotic medication (535.4 vs. 308.1 mg/day). Our results suggest that general levels of function and disability as assessed by family members using the LSP in outpatients with schizophrenia are associated with two types of symptomatology: depressive and negative symptoms.

In summery, we examined the relationship among patient-rated subjective QOL, observer-rated objective QOL, family-rated life skills, and their clinical determinants in outpatients with schizophrenia using schizophrenia disease-specific QOL measures as well as the LSP. The results indicate that depressive symptoms predict subjective QOL, negative symptoms predict objective QOL, and each of them predicts the level of social skills. Only the motivation/energy aspect, but no other aspect of subjective QOL, correlated with objective QOL. Family-rated life skills showed significant correlations with both subjective and objective QOL. These results suggest that the patient's QOL could be predicted by life skills assessed by a family member and also imply the importance of active treatment for depressive and negative symptoms in improving QOL and life skills of outpatients with schizophrenia.

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Regular Article

Pharmacokinetic interaction between tandospirone and fluvoxamine in the rat contextual conditioned fear stress model and its functional consequence: Involvement of cytochrome P450 3A4

Hiroyuki Nishikawa, PhD,[1]* Takeshi Inoue, MD, PhD,[1] Takuya Masui, MD,[1] Takeshi Izumi, MD, PhD,[1,2] Shin Nakagawa, MD, PhD,[1] and Tsukasa Koyama, MD, PhD[1]

Departments of [1]Psychiatry and [2]Neuropharmacology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Aims: In a previous study it was demonstrated that the anxiolytic action of tandospirone, a 5-hydroxytryptamine (5-HT)$_{1A}$ receptor agonist, is facilitated by cytochrome P450 (CYP) 3A4 inhibitors, such as ketoconazole and cimetidine. It is also known that fluvoxamine, a selective serotonin re-uptake inhibitor (SSRI), inhibits CYP3A4. The purpose of the present study was to clarify the pharmacokinetic interaction between tandospirone and fluvoxamine and to evaluate their combined effect in the rat anxiety model.

Methods: The anxiolytic action of co-administration of tandospirone and fluvoxamine was examined using the rat contextual conditioned fear stress model. After testing the conditioned fear, plasma concentrations of tandospirone and its major metabolite 1-(2-pyrimidyl) piperazine were determined.

Results: One day after fear conditioning, both tandospirone (60 mg/kg, p.o.) and fluvoxamine (60 mg/kg, p.o.) significantly inhibited conditioned freezing and their combination effect was additive. In addition, plasma concentration of tandospirone was increased by fluvoxamine.

Conclusions: There is a CYP3A4-related drug–drug interaction between tandospirone and fluvoxamine. Therefore, fluvoxamine may facilitate the anxiolytic effect of tandospirone via CYP3A4 inhibition.

Key words: conditioned fear, cytochrome P450 3A4, drug interaction, fluvoxamine, tandospirone.

TANDOSPIRONE, AN AZAPIRONE derivative with serotonin-1A (5-HT$_{1A}$) receptor agonistic action, is used in Japan and China and is similar to buspirone and ipsapirone, the US and European equivalents. These 5-HT$_{1A}$ receptor agonists are used for the treatment of generalized anxiety disorder.[1–4]

It is known that azapirones are mainly metabolized by cytochrome P450 (CYP) 3A4 isoforms.[5–8] Tandospirone is also a substrate of the CYP3A4 isoforms. Several groups have reported that tandospirone is primarily metabolized by CYP3A4 isoforms, and to a lesser extent by CYP2D6, in human liver microsomes.[7,8] In addition we have previously shown that plasma concentrations of tandospirone and buspirone in rats are increased by co-administration of CYP3A4 inhibitors such as ketoconazole and cimetidine, and that the anxiolytic action of these drugs, as evaluated by contextual conditioned freezing, is facilitated via CYP3A4-related drug–drug interaction.[9]

In addition to known CYP3A4 inhibitors, fluvoxamine, a selective serotonin re-uptake inhibitor (SSRI), also possesses moderate CYP3A4 inhibitory

Correspondence: Hiroyuki Nishikawa, PhD, Department of Psychiatry, Hokkaido University Graduate School of Medicine, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan. Email: hnishika@med.hokudai.ac.jp

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activity, although it is well known as a potent CYP1A2 and CYP2C19 inhibitor.[10–12] SSRI are widely used as first-line drugs for treatment of anxiety and major depressive disorders. If patients fail to respond to SSRI treatment, however, 5-HT_{1A} receptor agonist (i.e. tandospirone or buspirone) augmentation of SSRI might be performed as one of the augmentation strategies.[13–15] Therefore, co-administration of an azapirone and an SSRI with CYP3A4 inhibitory activity may result in drug–drug interaction. Indeed, interaction between buspirone and fluvoxamine in human healthy volunteers has been reported,[16,17] but drug–drug interaction between tandospirone and fluvoxamine has not been clarified. In the present study we investigated the effects of fluvoxamine on plasma concentration of orally administered tandospirone and evaluated the combinative action of these drugs in the rat contextual conditioned fear stress model as a functional consequence.

METHODS

Animals

Male Sprague–Dawley rats (250–350 g), obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan), were housed in groups of four in a temperature-controlled environment (22 ± 1°C) with free access to food and water. The animals were maintained on a 12-h light/dark cycle (light phase: 06:30–18:30) and tested during the light phase after 1-week acclimatization period. Thirty-two rats were tested for contextual conditioned fear stress (vehicle, tandospirone, fluvoxamine, tandospirone + fluvoxamine; $n = 8$). The tandospirone ($n = 8$) and tandospirone + fluvoxamine ($n = 8$) groups, after testing for conditioned fear stress, were used for the determination of plasma concentration of tandospirone. In a separate experiment, 32 rats were tested for motor activity (vehicle, tandospirone, fluvoxamine, tandospirone + fluvoxamine; $n = 8$)

All experiments were approved by the Hokkaido University School of Medicine Animal Care and Use Committee, and were in compliance with the Guide for the Care and Use of Laboratory Animals.

Drugs

Tandospirone citrate (a gift from Dainippon Sumitomo Pharma, Osaka, Japan) and fluvoxamine maleate (a gift from Solvay Pharmaceuticals, Weesp, The Netherlands) were dissolved in 0.9% sterile saline.

Contextual conditioned fear stress

The rats were individually subjected to inescapable electric footshocks for a total of 2.5 min (five footshocks [2.5-mA scrambled shock, 30 s duration] delivered at intershock intervals of 35–80 s [mean, 60 s]) in a shock chamber with a grid floor (19 × 22 × 20 cm; Medical Agent, Kyoto, Japan). Electric shocks were produced by a shock generator (Model SGS-02D, Medical Agent). One day after footshocks, the rats were again placed in the shock chamber without footshocks and observed for 5 min. During the observation period (i.e. testing) the duration of freezing behavior was recorded using a time-sampling procedure.[18] Every 10 s the behavior that the animal was currently engaged in was classified as either freezing or activity. Freezing was defined as the absence of all observable movement of the skeleton and the vibrissae, except those related to respiration. All other behavior was scored as activity. The animal was classified as either freezing or active according to its behavior throughout the entire 10-s period. The percentage freezing score (freezing (%)) represented the number of entire 10-s periods for which the animal froze.

Drug administration

One day after footshocks, the rats received a single oral administration of tandospirone citrate (60 mg/kg) 1 h before testing. Fluvoxamine maleate (60 mg/kg) was orally administered 4 h before testing (i.e. 3 h prior to administration of tandospirone citrate). Tandospirone citrate and fluvoxamine maleate were given in a volume of 5 mL/kg.

Determination of tandospirone and its major metabolite (1-[2-pyrimidyl] piperazine) concentrations in plasma

After testing, the rats were immediately decapitated and the blood was collected into heparin-containing tubes. Plasma samples were prepared by centrifuging the blood samples at 1000 *g* for 15 min and stored at −20°C until analysis. The free base concentrations of tandospirone and 1-(2-pyrimidyl) piperazine (1-PP) in the plasma samples were determined using liquid chromatography with a tandem mass spectrometry

(LC/MS/MS) system. An appropriate internal standard solution (50 μL) and distilled water (550 μL) were added to each plasma sample (100 μL) and mixed thoroughly. The mixture was then applied to a solid-phase extraction cartridge (Oasis HLB 60 mg/3 mL, Waters Corporation, Tokyo, Japan) preconditioned with water. The cartridge was washed with water (2 mL) and elution objectives were performed using methanol (3 mL). Following addition of 2% (v/v) propylene glycol (500 μL), the samples were refluxed to dryness under N_2 gas and the residues were dissolved in 10 mmol/L ammonium acetate (500 μL). A total of 20 μL of each sample was injected into a high-performance liquid chromatography (HPLC; 10A, Shimazu, Kyoto, Japan) with a YMC Hydrosphere C18 column (5 μm particle size, 75 × 2.0 mm, YMC, Kyoto, Japan). For analysis of tandospirone and 1-PP, the mobile phase consisted of 10 mmol/L ammonium acetate (solvent A) and methanol (solvent B). Initially, the mobile phase consisted of 80% A and 20% B and then changed to 20% A and 80% B with a linear gradient over 30 s and a flow rate of 0.3 mL/min. Tandospirone and 1-PP were assayed on an MS/MS system using positive-ion electrospray ionization (API4000, Sciex, Toronto, Ontario, Canada). The lower limits for quantification of tandospirone and 1-PP were 0.1 ng/mL and 1 ng/mL, respectively. The standard curves for tandospirone and 1-PP were linear up to 50 ng/mL and 500 ng/mL, respectively. If the measured value of tandospirone or 1-PP plasma concentration was not linear on the standard curve, the sample was diluted with blank plasma and re-assayed.

Measurement of motor activity

Rats motor activity was measured for tandospirone (60 mg/kg, p.o.), fluvoxamine (60 mg/kg, p.o.), and their co-administration. The rats were habituated to the testing room within their housing cages for 1 day. Tandospirone and fluvoxamine were administered 1 h and 4 h prior to testing, respectively. In a separate experiment, rats received co-administration of tandospirone and fluvoxamine. During testing, rats were individually subjected to the testing cage and motor activity was automatically recorded for 5 min by an infrared sensor that detected thermal radiation from the animals.[19] Horizontal movements were digitized and fed into a computer. Locomotion predominantly contributed to the count, but other body movements also contributed when they contained substantial horizontal components.

Data analysis

All data are presented as mean ± SEM of individual values for each rat in all groups. Statistical analysis was performed using Welch test for two groups, or two-way ANOVA for the drug–drug interaction.

RESULTS

Effects of co-administration of tandospirone and fluvoxamine on rat conditioned fear stress

Previous studies have shown that acute administration of tandospirone (30–100 mg/kg, p.o.) and fluvoxamine (30–100 mg/kg, p.o.) dose-dependently reduces conditioned freezing 1 day after fear conditioning in rats.[9,20] In accordance with these studies, the doses of tandospirone (60 mg/kg, p.o.) and fluvoxamine (60 mg/kg, p.o.) were chosen as the minimal effective doses.

Figure 1 shows the combined effect of tandospirone (60 mg/kg, p.o.) and fluvoxamine (60 mg/kg, p.o.) on rat conditioned freezing. Two-way

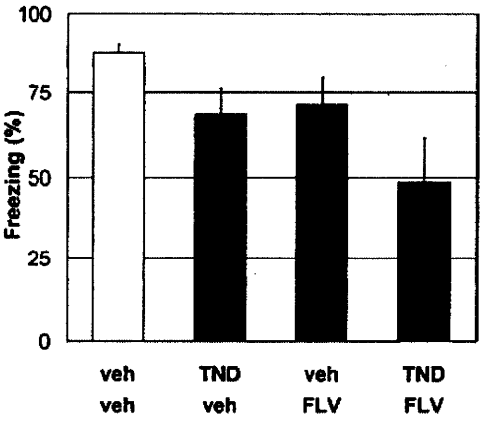

Figure 1. Effect of co-administration of tandospirone and fluvoxamine on contextual conditioned fear stress in rats. One day after footshocks, tandospirone (TND; 60 mg/kg, p.o.) and fluvoxamine (FLV; 60 mg/kg, p.o.) were administered 1 h and 4 h before testing. There was a significant main effect of TND and a nearly significant main effect of FLV, but no interaction. $n = 8$. Veh, vehicle (0.9% sterile saline).

Table 1. Effects of fluvoxamine on plasma concentrations of tandospirone and 1-PP in rats (mean ± SEM, $n = 8$)

Treatment (mg/kg)	TND (ng/mL)	1-PP (ng/mL)	1-PP/TND ratio
TND citrate (60) + vehicle	6.4 ± 0.4	345.6 ± 29.3	54.0 ± 3.8
TND citrate (60) + FLV (60)	12.5 ± 2.5*	276.6 ± 17.8**	27.6 ± 4.4***

*$P < 0.05$ vs TND citrate (60 mg/kg) alone; **$P = 0.067$ vs TND citrate (60 mg/kg) alone; ***$P < 0.001$ vs TND citrate (60 mg/kg) alone.
TND was administered as a salt (TND citrate) and detected as the free base.
1-PP, 1-(2-pyrimidyl) piperazine; FLV, fluvoxamine; TND, tandospirone.

ANOVA indicated a significant main effect of tandospirone ($F(1, 28) = 5.65$, $P < 0.05$) and a weak effect of fluvoxamine ($F(1, 28) = 4.19$, $P = 0.0501$). No interaction between tandospirone and fluvoxamine was observed ($F(1, 28) = 0.03$, $P = 0.870$).

Effect of fluvoxamine on the plasma concentrations of tandospirone and its major metabolite, 1-PP

After testing, rats were immediately decapitated and blood was collected to determine plasma concentrations of tandospirone and its major metabolite, 1-PP. As shown in Table 1, plasma concentration of tandospirone was significantly higher in the tandospirone + fluvoxamine-treated group than in the tandospirone alone-treated group ($P < 0.05$, Welch test). Accompanying the increase in plasma concentration of tandospirone, plasma concentrations of 1-PP was slightly, but not significantly, lower in the tandospirone + fluvoxamine-treated group than in the tandospirone alone-treated group ($P = 0.067$, Welch test). Moreover, 1-PP/tandospirone ratio, an index of tandospirone metabolism, was significantly reduced in the tandospirone + fluvoxamine-treated group as compared to the tandospirone alone-treated group ($P < 0.001$, Welch test).

Motor activity

Table 2 shows the combined effect of tandospirone (60 mg/kg, p.o.) and fluvoxamine (60 mg/kg, p.o.) on rat motor activity. Two-way ANOVA indicated significant main effects of tandospirone ($F(1, 28) = 7.69$, $P < 0.01$) and fluvoxamine ($F(1, 28) = 7.49$, $P < 0.05$). No interaction between tandospirone and fluvoxamine was observed ($F(1,28) = 1.17$, $P = 0.288$).

DISCUSSION

In the present study fluvoxamine increased plasma concentration of tandospirone, probably via CYP3A4 inhibition. In accordance with this increase, co-administration of tandospirone and fluvoxamine additively reduced conditioned freezing in rats. These findings suggest that CYP3A4-related drug–drug interaction between tandospirone and fluvoxamine may positively affect their anxiolytic action.

Moreover, we found that co-administration of tandospirone and fluvoxamine additively reduces motor activity in rats. It has been reported that high doses (160–320 mg/kg, p.o.) of tandospirone reduce spontaneous motor activity in mice.[21] In addition, we have shown in a previous study that co-administration of tandospirone and a CYP3A4 inhibitor (i.e. ketoconazole or cimetidine) reduces motor activity in rats.[9] Nevertheless, because two-way ANOVA showed that both tandospirone and fluvoxamine significantly reduced rat motor activity in the present study and no interaction was observed between the two drugs, we could not conclude that the reduced motor activity in the combination group is due to increased plasma

Table 2. Effect of co-administration of tandospirone and fluvoxamine on spontaneous locomotor activities (mean ± SEM, $n = 8$)

Treatment (mg/kg)	Locomotor activity (arbitrary unit)
Vehicle + vehicle	1044.8 ± 101.9
TND (60 mg/kg, p.o.) + vehicle	696.9 ± 88.9
Vehicle + FLV (60 mg/kg, p.o.)	700.0 ± 80.2
TND (60 mg/kg, p.o.) + FLV (60 mg/kg, p.o.)	547.5 ± 88.6

There were significant main effects of tandospirone and fluvoxamine, but no interaction.
FLV, fluvoxamine; TND, tandospirone.

tandospirone concentration. These results, however, strongly suggest that the inhibition of rat conditioned freezing observed with co-administration of tandospirone and fluvoxamine is not a false-positive effect caused by increased motor activity.

In the present study both tandospirone and fluvoxamine were orally administered to clarify their drug–drug interaction. Interestingly, we found in a previous study that co-administration of s.c. tandospirone (0.3 mg/kg) and i.p. fluvoxamine (30 mg/kg), given at subeffective doses, markedly reduced conditioned freezing in rats.[22] In that case, however, plasma concentration of tandospirone was never increased by fluvoxamine, indicating that the enhanced anxiolytic effect observed in the combination group appeared without affecting CYP3A4-related pharmacokinetic drug–drug interaction. Moreover, co-administration of s.c. tandospirone and i.p. paroxetine or citalopram, two SSRI with no CYP3A4 inhibitory activity, significantly reduces conditioned freezing in rats as compared with the vehicle or individual SSRI.[22] These findings elucidate the pharmacodynamic synergistic effect of tandospirone and individual SSRI, which is probably via stimulation of the post-synaptic 5-HT_{1A} receptor.[22] Dissimilar to our previous study (i.e. a combination of s.c. tandospirone and i.p. fluvoxamine), oral co-administration of tandospirone and fluvoxamine significantly increased plasma concentration of tandospirone. The inconsistency between these alterations of plasma tandospirone may be caused by tandospirone's pharmacokinetic properties related to absorption and metabolism. It is known that azapirones, including tandospirone, are rapidly absorbed and undergo extensive first-pass metabolism after oral administration.[23] Therefore, orally administered tandospirone might be more easily affected by CYP3A4 inhibitors than s.c. tandospirone. Indeed, our previous study showed that plasma concentrations of s.c. tandospirone were hardly affected by the CYP3A4 inhibitor ketoconazole in rats.[22] These findings suggest that both pharmacokinetic and pharmacodynamic drug–drug interactions contribute to the combined effect of orally administered tandospirone and fluvoxamine.

In contrast, the enhancement of anxiolytic effects of oral tandospirone by fluvoxamine seemed to be weak, considering both pharmacokinetic and pharmacodynamic drug–drug interactions, because two-way ANOVA on the anxiolytic effect indicated no interaction between tandospirone and fluvoxamine. We could not exclude the possibility that reduced motor activity might have masked the inhibitory effects on rat freezing behavior. In addition, methodological limitations (i.e. we measured both pharmacological behavior and plasma concentrations at a single time point and at a single combination dose) might have influenced the results. Therefore, further studies on the time- and dose-dependent pharmacokinetic and pharmacodynamic interactions between tandospirone and fluvoxamine are needed. The finding that the anxiolytic effect of tandospirone and fluvoxamine combination was additive is useful for estimation of drug–drug interaction in humans. We should pay attention to these drug–drug interactions; in particular, the dose of tandospirone or other azapirones should be sufficiently reduced during concomitant treatment with fluvoxamine. Further studies on drug–drug interaction between tandospirone and fluvoxamine in humans are needed.

CONCLUSIONS

In the present study we investigated the combined effect of tandospirone and fluvoxamine in the rat contextual conditioned fear stress model. Co-administration of oral tandospirone and fluvoxamine additively reduced conditioned freezing in the rat. This effect was accompanied by an increase in plasma concentration of tandospirone. These results indicate pharmacokinetic drug–drug interaction between tandospirone and fluvoxamine. Therefore, the anxiolytic effect of tandospirone may be facilitated by fluvoxamine via CYP3A4-related drug–drug interaction.

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Changes in amygdala neural activity that occur with the extinction of context-dependent conditioned fear stress

Takeshi Izumi [a,b,*], Takeshi Inoue [b], Akiko Kato [b], Yuji Kitaichi [b], Shin Nakagawa [b], Tsukasa Koyama [b]

[a] Department of Neuropharmacology, Hokkaido University Graduate School of Medicine, North 15, West 7, Sapporo 060-8638, Japan
[b] Department of Psychiatry, Hokkaido University Graduate School of Medicine, North 15, West 7, Sapporo 060-8638, Japan

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ABSTRACT

The purpose of the present study was to characterize functional changes in the amygdala that accompany the extinction of context-dependent conditioned fear stress in a rat, an animal model of anxiety. Specifically, the effect of extinction of conditioned fear-induced cyclic AMP responsive element-binding protein (CREB) phosphorylation in the amygdala was investigated using immunohistochemistry. Experiments demonstrated that CREB phosphorylation in the basal nucleus of the amygdala decreased with the extinction of context-dependent conditioned fear-induced freezing behavior. These data suggest that the basal nucleus of the amygdala plays an essential role in the expression of context-dependent conditioned fear. Further, this is the first study to demonstrate that CREB phosphorylation in the basal nucleus of the amygdala changes in parallel with the extinction of context-dependent conditioned fear.

1. Introduction

Past studies have demonstrated that the amygdala plays a crucial role in anxiety and fear (Ono and Nishijo, 1992; LeDoux, 2000) and that the amygdala may be a target for the action of various kinds of anxiolytic drugs (Beck and Fibiger, 1995; Menard and Treit, 1999; Inoue et al., 2004). We recently reported that conditioned fear stress (CFS), an animal model of anxiety in rats, specifically induced c-Fos expression in the basal nucleus of the amygdala and that the administration of citalopram, a selective reuptake inhibitor, attenuated this increase in c-Fos expression (Izumi et al., 2006).

Conditioned fear stress is a type of classical conditioning (Fanselow, 1980) distinguished by acquisition, expression, and extinction (Myers and Davis, 2002). Acquisition occurs when a sensory stimulus (CS, conditioned stimulus), such as light, tone, or exposure to the test box (context), is paired with an aversive stimulus (US, unconditioned stimulus), such as footshock. Expression occurs when the animal is re-exposed to the CS without the US, and it elicits a variety of autonomic, hormonal, and behavioral conditioned responses. Extinction occurs when the CS is repeatedly presented in the absence of the US, and it decreases the amplitude of conditioned responses.

Extinction is thought to be an active learning process (Myers and Davis, 2002). Several studies have attempted to characterize the effect of a prefrontal cortex lesion on extinction, but the results have varied (Gewirtz et al., 1997; Morgan and LeDoux, 1999; Quirk et al., 2000). Further, administration of a *N*-methyl-D-aspartate (NMDA) receptor glycine site agonist facilitated extinction, while administration of a NMDA receptor antagonist, benzodiazepine receptor agonist, benzodiazepine receptor inverse agonist, muscarinic receptor antagonist, or dopamine-1 receptor agonist inhibited extinction (reviewed by Myers and Davis, 2002; Davis and Myers 2002).

The goal of the present study was to characterize changes in the amygdala neural activity that occur with the extinction of context-dependent CFS, using cAMP responsive element-binding protein (CREB) phosphorylation as an index of cellular activity.

2. Methods

This study was approved by the Hokkaido University School of Medicine Animal Care and Use Committee, and all protocols complied with the Guide for the Care and Use of Laboratory Animals of the Hokkaido University School of Medicine.

2.1. Animals

Male Sprague–Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), weighing 250–300 g, were used. Four rats were housed per cage (38×33×17 cm), in a 12-h light:12-h dark cycle and a temperature-controlled environment (22±1 °C) with free access to food and water. Experiments were initiated after a 14-day adaptation period.

* Corresponding author. Department of Neuropharmacology, Hokkaido University Graduate School of Medicine, North 15, West 7, Sapporo 060-8638, Japan. Tel.:+81 11 706 5058; fax: +81 11 706 7872.
E-mail address: psyizumi@med.hokudai.ac.jp (T. Izumi).

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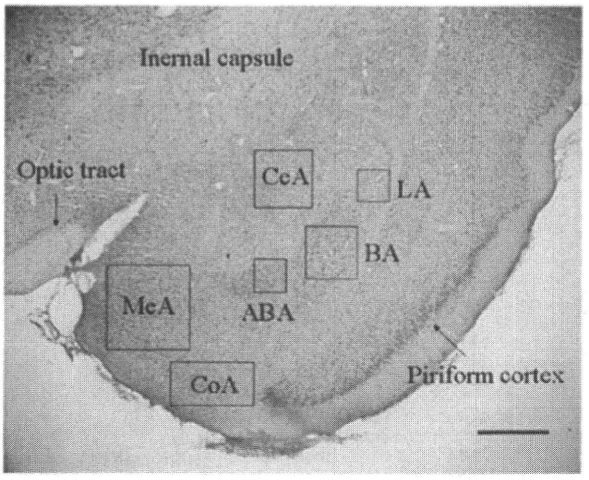

Fig. 1. Nissl staining of the amygdala (−3.14 mm to Bregma). BA, basal nucleus of the amygdala; ABA, accessory basal nucleus of the amygdala; CeA, central nucleus of the amygdala; CoA, cortical nucleus of the amygdala; LA, lateral nucleus of the amygdala; MeA, medial nucleus of the amygdala. Bar=500 μm.

2.2. CFS-induced freezing

Each rat was placed in a shock chamber (19×22×20 cm) and underwent 5 min of inescapable electric shocks (scrambled shocks of 0.2-mA intensity and 30-s duration, five times at variable intervals). Twenty-four hours after the footshock, the rats were again placed in the shock chamber and observed for 5 min without any shock application. During the 5-min observation period, freezing behavior was recorded using a time-sampling procedure (Fanselow, 1980), in which the animal behavior was classified as either "freezing" or "activity" at every 10-s interval. Freezing was defined as the lack of any observable movement of the body and the vibrissae, with the exception of movements related to respiration. Percentage scores for freezing were calculated for a 5-min observation period. Analysis of the freezing behavior was performed by an investigator who was blinded to the treatment.

2.3. Immunohistochemistry

Rats were anesthetized by pentobarbital injection (40 mg/kg, intraperitoneally) and perfused with saline and then by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brains were sectioned at 30-μm thickness. Immunohistochemistry was performed on free-floating coronal sections (Umino et al., 1995). After 24-h incubation in 0.01 M phosphate-buffered saline and normal goat serum, the sections were incubated for 48 h in 0.01 M phosphate-buffered saline containing 0.2% Triton X-100 and rabbit anti-phospho-CREB antibody (Upstate Biotechnology, NY, 1:1000 dilution). The sections were incubated for 1 h in 0.01 M phosphate-buffered saline containing 0.2% Triton X-100 and biotinylated goat anti-rabbit IgG (Vector Labs) and then were incubated for 1 h in 0.01 M phosphate-buffered saline and avidin–biotinylated horseradish peroxidase complex (Vector Labs, Vectastain Elite ABC Kit). The reaction product was visualized by transferring the sections to a 50 mM Tris–HCl buffer (pH 7.6) containing 0.05% diaminobenzidine, 0.6% nickel ammonium sulfate and 0.01% H_2O_2.

2.4. Semiquantitative cell counting

According to the atlas of Paxinos and Watson (1997), the section that was located −3.14 mm posterior from the bregma was selected for semiquantitative evaluation of phospho-CREB (pCREB) immunoreactivity with a densitometric video image analysis system (MCID system, Imaging Research, CA, USA), according to the method of Bilang-Bleuel et al. (2002). The unit areas (200×200 μm) of the lateral nucleus, basal nucleus, accessory basal nucleus, central nucleus, medial nucleus, and cortical nucleus of the amygdala (Fig. 1) were digitally recorded by a CCD camera (CCD-IRIS, Sony, Japan) connected to a photomicroscope (B× 50, Olympus, Japan). The number of pCREB positive cells was assessed by automated selection of those cells within the unit areas that satisfied the following criteria: (1) the gray value of the cell nucleus was higher than the threshold value (threshold gray

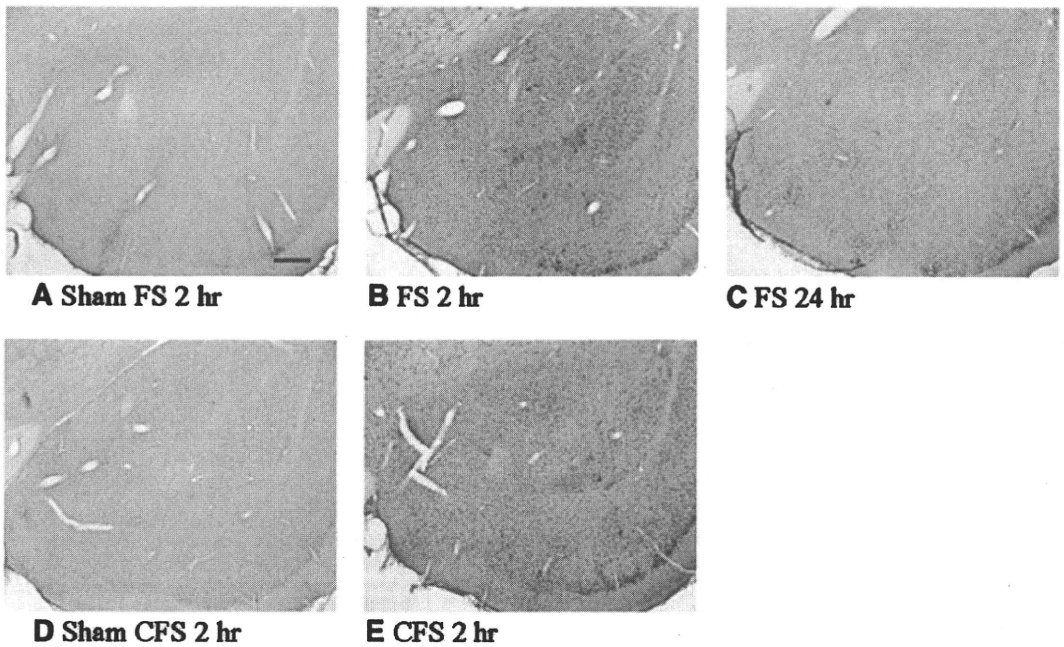

Fig. 2. Photomicrographs of the amygdala showing the expression of footshock and conditioned fear stress-induced phosphorylated CREB-like immunoreactivity. (A) 2 h after sham FS; (B) 2 h after FS; (C) 24 h after FS; (D) 2 h after sham CFS; (E) 2 h after CFS. FS, footshock; CFS, conditioned fear stress. Bar=200 μm.