were detected by PCR–RFLP methods as described by Tanabe et al. (2001).

### 2.5. Assay

Plasma concentrations of bromperidol and reduced bromperidol were determined in duplicate using the high-performance liquid chromatographic (HPLC) methods developed in our laboratory (Yasui-Furukori et al., 2004a). The lowest limit of detection was 1.0 ng/ml, and interassay coefficient of variation (CV) was less than 5.1% for both compounds.

### 2.6. Data analyses and statistics

The 18 items in BPRS were divided into five subgroups, i.e., positive (exaggerated self-esteem, suspiciousness, hallucinations and unusual thought content), excitement (hostility, uncooperativeness and psychomotor agitation), cognitive (conceptual disorganization, specific motor disturbance and disorientation), negative (emotional withdrawal, psychomotor retardation and blunted affect) and anxiety–depression (somatic concern, anxiety [psychic], self-depreciation, anxiety [somatic] and depressive mood) symptoms as classified by Lindenmayer et al. (1995). The UKU items were also divided into three subgroups, i.e., psychic (concentration difficulties, asthenia, sleepiness, failed memory and depression), extrapyramidal (dystonia, rigidity, hypokinesia, hyperkinesia, tremor, akinesia and increased salivation) and autonomic (accommodation disturbances, reduced salivation, constipation, micturation disturbance, orthostatic dizziness and palpitation) side effects. A pretreatment score was subtracted from one obtained during the treatment and was recorded as drug-associated side effect. Scores of sub-grouped side effects after 3-week treatment were used for statistical analysis. In only analysis of EPSs, the data in patients treated with biperiden were excluded after administration of the medication.

The comparisons of severity of illness (baseline BPRS scores), age, body weight, duration of illness and plasma concentrations of bromperidol and reduced bromperidol between *MDR1* genotypes were made by ANOVA followed by Tukey's test. Multiple regression analyses were conducted for correlations of age, gender, drug concentrations, co-administration of biperiden and *MDR1* genotypes with % improvement in 5 sub-grouped BPRS symptoms and UKU scores of 3 sub-grouped side effects. Dummy variables (male=0 and female=1) were used for analysis of gender effects. Because *C3435T* (Hoffmeyer et al., 2000) and *G2677T/A* (Siegmund et al., 2002) genotype proportionately expressed P-glycoprotein in intestine, dummy variables were used for analyses of *MDR1* genotype effects as follows: *CC*=2, *CT*=1 and *TT*=0 for *C3435T*, and *GG*=2, *GA* or *TA*=1 and *TT*, *TA* or *AA*=0 for *G2677T/A*. Effects of antiparkinson agents on therapeutic response were compared using *t*-test. A $p$ value less than 0.05 was regarded as statistically significant. All analyses were performed using SPSS 12.0J for windows (SPSS Japan Inc., Tokyo, Japan).

## 3. Results

Patients were *C/C* in 12, *C/T* in 12 and *T/T* in 7 cases for *C3435T* genotype and *G/G* in 3, *G/T* or *A* in 17 and *T* or *A/T* or *A* in 11 cases for *G2677T/A* genotype. There was no difference in clinical profiles such as age, gender or duration of illness between *C3435T* genotypes or between *G2677T/A* genotypes. Since significant difference in baseline BPRS scores was found between *C3435T* genotypes, statistical significance both in percentage improvement and improved scores were defined as clinically relevant findings.

Table 1
Clinical profiles, drug therapy and percent improvement in *C3435T* and *G2677T/A* genotypes

| | *C3435T* | | | *G2677T/A* | | |
|---|---|---|---|---|---|---|
| | *C/C* ($n$=12) | *C/T* ($n$=12) | *T/T* ($n$=7) | *G/G* ($n$=3) | *G/T* or *A* ($n$=17) | *T* or *A/T* or *A* ($n$=11) |
| Clinical profile | | | | | | |
| Age (years) | 37±12 | 34±13 | 41±13 | 46±9 | 34±13 | 39±13 |
| Gender (M/F) | 1 | 5/7 | 6/1 | 1/2 | 10/7 | 6/5 |
| Duration of illness (month) | 108±100 | 115±100 | 158±107 | 216±113 | 85±71 | 154±116 |
| Baseline BPRS score | 29±6 | 22±6 | 25±5* | 31±2 | 25±8 | 24±5.1 |
| Drug therapy | | | | | | |
| Dose (mg/day) | 11.4±5.0 | 9.3±3.9 | 16.3±2.7* | 8.0±2.8 | 10.7±4.6 | 14.2±4.6 |
| Plasma concentration (ng/ml) | 6.7±5.0 | 5.7±3.1 | 11.1±2.9* | 7.2±4.6 | 6.3±4.2 | 9.0±4.3 |
| Prolactin response (ng/ml) | 15.2±13.9 | 11.4±7.9 | 13.7±8.9 | 9.1±6.5 | 14.3±12.8 | 13.2±8.1 |
| Percent improvement | | | | | | |
| Total BPRS score | 61±32 | 56±29 | 47±27 | 79±14 | 62±30 | 41±26 |
| Positive | 63±38 | 56±42 | 64±30 | 87±18 | 65±35 | 47±42 |
| Excitement | 74±30 | 77±26 | 61±47 | 83±24 | 76±27 | 63±44 |
| Cognitive | 89±21 | 85±33 | 19±104 | 100±0 | 89±20 | 35±92 |
| Negative | 47±52 | 32±51 | 39±38 | 72±21 | 48±46 | 16±51 |
| Anxiety–deperssion | 34±41 | 41±46 | 29±13 | 56±14 | 37±47 | 24±24 |

Data are shown as mean±S.D.
* $p<0.01$ among three *C3435T* genotypes.

Although there were significant differences in clinical profiles such as daily dose or plasma concentration of bromperidol between *C3435T* genotypes ($p<0.05$) and post hoc analyses showed significant differences in *C/T* vs. *T/T*, there were no differences in the percentage improvement or the improved scores of total BPRS scores or 5 sub-grouped symptoms after the 3-week treatment between *C3435T* genotypes (Table 1). Clinical profiles did not differ between *G2677T/A* genotypes, while significant difference in the improved score of total BPRS was found between *G2677T/A* genotypes and post hoc analyses showed significant differences in *G/G* vs. *T* or *A/T* or *A*. There were no differences in the percentage improvement or the improved scores of 5 sub-grouped symptoms after the 3-week treatment between *G2677T/A* genotypes (Table 1). Furthermore, no differences were found in side effects induced by bromperidol between *C3435T* genotypes or between *G2677T/A* genotypes.

Multiple regression analyses including age, body weight, gender, drug concentration and *MDR1* genotypes (*C3435T* and *G2677T/A*) showed significant correlations between *C3435T* genotypes and the percentage improvement or the improved scores of cognitive symptoms (Table 2). There were significant correlations between age and improved score of excitement symptoms and between body weight and percent improvement of excitement symptoms and between age and percent improvement of negative symptoms. No correlations were found in other symptoms vs. other variables. Neither total side effect nor sub-grouped side effects correlated with any clinical profiles including *MDR1* genotypes.

There were no differences in percent improvement or improved scores of total BPRS and 5 clusters including cognitive symptoms between subjects treated with biperiden and without biperiden.

## 4. Discussion

P-glycoprotein is found in the epithelial cells lining the luminal surface of many organs often associated with an excretory or barrier function, that is, the hepatic bile canalicular membrane, renal proximal tubule, villus-tip enterocyte in the small intestine, and the endothelial cells making up the blood–brain and blood–testes barriers (Cordon-Cardo et al., 1989). Oral administration of [$^3$H] ivermectinin in the knockout animal, *mdr1a* (−/−) mice and mdr1a (+/+) mice resulted in 87-fold higher levels of radioactivity in the brain of *mdr1a* (−/−) mice as compared with wild-type mice, whereas the levels in liver, kidney, small intestine and plasma were increased by less than 4-fold (Rao et al., 1999). This finding suggests that P-glycoprotein in blood–brain and blood–testes barriers has major impact on the disposition of P-glycoprotein substrates.

The results of this study showed that there were no correlations between *C3435T* variants and total BPRS score or 5 sub-grouped scores using simple correlation analyses. In cognitive impairment, patients with *TT* type had a trend of poor improvement as evaluated with improved scores and percentage improvement, although these were not statistically significant because of relatively large standard deviation. However, multiple regression analyses including plasma drug concentration showed that *C3435T* variant had significant association between the impaired scores and percentage improvement of cognitive symptoms. If lower expression and function of P-glycoprotein was present in blood–brain barrier in *TT* type as well as small intestine, a lower efflux function for bromperidol and hence higher drug concentration in brain might occur in *TT* type. In the light of the hypothesis that typical antipsychotic agents lead to worsening of cognitive function, higher drug concentration in brain might be associated with poor efficacy on cognitive

Table 2
Standard partial correlation coefficients between clinical profiles including MDR1 genotypes and percentage improvement or improved scores in the treatment with bromperidol

| Variables | Standard partial correlation coefficients | | | | |
|---|---|---|---|---|---|
| | Positive | Excitement | Cognitive | Negative | Anxiety–Depression |
| Percentage improvement | | | | | |
| Gender | −0.257 | 0.253 | 0.094 | −0.052 | 0.053 |
| Age | 0.372 | 0.326 | 0.224 | −0.490* | 0.325 |
| Body weight | 0.184 | 0.569* | 0.286 | 0.060 | 0.198 |
| *C3435T* | 0.075 | 0.219 | 0.464* | −0.064 | −0.083 |
| *G2677T/A* | 0.162 | 0.101 | −0.003 | 0.265 | 0.220 |
| Drug concentration | 0.017 | 0.020 | 0.030 | 0.031 | 0.085 |
| Improved scores | | | | | |
| Gender | −0.390 | 0.010 | −0.394 | −0.226 | −0.182 |
| Age | 0.392 | 0.465* | 0.134 | −0.295 | 0.107 |
| Body weight | 0.099 | 0.310 | −0.006 | 0.049 | 0.038 |
| *C3435T* | 0.171 | 0.293 | 0.673** | 0.358 | 0.034 |
| *G2677T/A* | 0.306 | −0.113 | −0.251 | −0.085 | 0.227 |
| Drug concentration | 0.090 | 0.171 | 0.050 | 0.059 | −0.138 |

* $p<0.05$.
** $p<0.01$.

function, although we had no evidence of higher drug concentration in the brain in *TT* type than other *MRD1* genotypes. However, the possibility that higher plasma drug concentration and hence excessive dopamine receptor blockade simply resulted in poor improvement of cognitive function cannot be excluded entirely, because significant difference in plasma bromperidol concentration was found between *C3435T* genotype in this study.

It is considered that large inter-individual variation in percentage improvement of cognitive function in this study led to lack of statistical difference between *C3435T* genotypes. The large inter-individual variation may be explained by data included by both patients with deterioration of cognitive function as side effect and patients with amelioration of cognitive function as efficacy, provided that the BPRS may have methodological problems.

A previous study suggested that a significant association was present between nortriptyline-induced postural hypotension and *C3435T* ($P$=0.034) in patients with major depression enrolled in a randomized antidepressant treatment trial of nortriptyline and fluoxetine (Roberts et al., 2002). The results suggest that homozygosity for *3435T* alleles is a risk factor for occurrence of nortriptyline-induced postural hypotension (OR=1.37, $P$=0.042, 95% CI: 1.01–1.86). These results support our finding because it is possible that postural hypotension is also induced by higher drug concentration in the brain.

Another single-nucleotide polymorphism in exon 21 of the *MDR1* gene (*G2677T/A*) is also linked with a lower function of P-glycoprotein (Siegmund et al., 2002). Improved scores in total BPRS scores correlated significantly with *G2677T/A* variants while no correlations were found between *G2677T/A* variants and any symptoms using multiple regression analyses. Therefore it is less likely that *G2677T/A* variants are useful for prediction of efficacy when bromperidol is used in the treatment of schizophrenia.

It is known that antagonists to acetylcholine such as biperiden or triphexyphnydil impair cognitive function (Tracy et al., 2001). However, there was no difference in percent improvement of cognitive function between subjects treated with biperiden and without biperiden. Thus it is unlikely that coadministration of anticholinergic agents plays an important role in treatment of cognitive impairment.

Our previous studies have shown that dopamine $D_2$ receptors (*DRD2*) polymorphisms such as *Taq IA* and *−141C Ins/Del* are associated with positive symptoms (Suzuki et al., 2000) and anxiety–depression symptoms (Suzuki et al., 2001b), respectively, in schizophrenic patients treated with selective *DRD2* antagonists. In addition, we have reported that neuroleptic malignant syndrome is also linked with these *DRD2* polymorphisms (Suzuki et al., 2001a). These findings suggest that several clinical responses to selective *DRD2* antagonists are dependent upon inter-individual variation of target receptor function.

An in vitro study has shown that quetiapine and risperidone have stronger affinity to P-glycoprotein than other atypical antipsychotic drugs, suggesting that quetiapine and risperidone are substrates of P-glycoprotein (Boulton et al., 2002). However, there is no in vivo data indicating that quetiapine or risperidone as a substrate of P-glycoprotein is of clinical relevance except an in vivo data showing lack of impact of *MDR1* genotype on steady-state plasma concentration of risperidone and 9-hydroxyrisperidone (Yasui-Furukori et al., 2004b). Further information is required for antipsychotics to determine whether these *MDR1* genotypes are clinically relevant or not.

## 5. Conclusion

The present results suggest that the *C3435T* polymorphism is associated with some therapeutic response to bromperidol in schizophrenic patients. This finding is possibly explained by different drug concentration in the brain between *MDR1* genotypes. However, further replication studies are required to determine whether or not *MDR1* genotypes are clinically relevant in the treatment with antipsychotics because this study had a relatively small number of subjects.

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# Case Report

## Digitalis intoxication induced by paroxetine co-administration

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In February, 2005, a 68-year-old woman, who had no history of psychiatric illness, presented to our department with a major depressive disorder, with moderate sadness, inner tension, difficulty in concentrating, severe sleep impairment, appetite loss, and pessimistic thoughts. She had atrial fibrillation (AF) which had been treated with 0·25 mg digoxin daily and 1 mg warfarin daily for 2 years (INR 1·7). There were no abnormal physiological findings except a slightly raised serum creatinine concentration (106 μmol/L). She was admitted to the psychiatric ward. On day 3 of her admission, we initiated paroxetine 20 mg/day for depression. Nausea, vomiting, and dizziness began on day 5. Delirium with visual hallucinations and disorientation developed on day 7. She was not able to eat or walk on day 10. On day 11, we suspected digitalis intoxication (serum digoxin concentration 5·2 ng/mL, normal range 0·5–2·0 ng/mL; ECG showed many ventricular premature contractions and complete A-V block). There was no electrolyte disturbance. All medications were discontinued from day 12. As a rebound effect of digoxin discontinuation, bradycardia was observed from day 13 to day 15, and subsequently the ECG showed AF as the plasma concentration of digoxin was decreasing.

On day 21, digoxin 0·25 mg daily and warfarin 1 mg daily were reinstated. The delirium with disorientation recovered gradually and ameliorated fully by day 28. However, slight to moderate depressive symptoms remained She became bedridden during these events and developed aspiration pneumonia due to difficulty in swallowing despite recovery of her appetite on day 45. Primary treatment for the pneumonia with antibiotics was started, but her physical condition did not change. In May, 2005, she was moved to a medical ward for intensive care, and she died from pneumonia in June, 2005. We measured plasma concentrations of digoxin retrospectively (figure).

Negative affective states such as depression are associated with premature mortality and increased risk of coronary heart disease, type 2 diabetes, and disability. Patients with chronic medical illness and comorbid depression show substantial improvements in mood, social and emotional functioning, and disability after initiation of antidepression treatment.[1] Patients scoring in a higher quartile for anxiety and total psychological distress are at a greater risk of developing AF than those in lower quartiles.[2] Therefore, patients are likely to be prescribed antidepressants concomitantly with digoxin. In our patient, there were no symptoms suggesting digitalis intoxication during digoxin treatment alone. Plasma concentrations of digoxin increased during administration of paroxetine. Several symptoms disappeared with the decrease in plasma concentration of digoxin, showing that

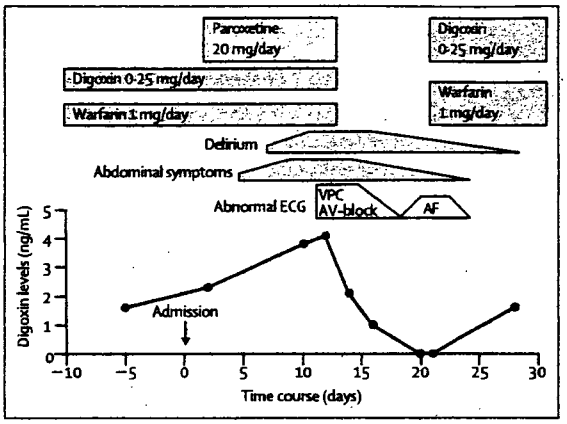


***Figure:*** **Clinical course of digitalis-paroxetine interaction**
VPC, ventricular premature contraction; AF, atrial fibrillation.

concomitant administration of paroxetine with digoxin caused the digitalis intoxication. Several in-vivo studies have consistently suggested that paroxetine decreases CYP2D6 activity, resulting in drug interactions,[3] and an in-vitro study showed potent P-glycoprotein inhibitory activity with paroxetine.[4] Digoxin is regarded as a substrate of P-glycoprotein on the basis of several interaction studies.[5] The high digoxin concentrations during paroxetine administration in our case probably resulted from P-glycoprotein inhibition in the kidney. Since nausea, vomiting, and reduced appetite are not only early symptoms of digitalis intoxication but also some of the side-effects of selective serotonin-reuptake inhibitors (SSRI), it is difficult to distinguish early symptoms of digitalis intoxication from SSRI-mediated side-effects or symptoms of depression. Therefore, therapeutic drug monitoring of digoxin concentrations is necessary in the early stage of concomitant administration with SSRI. In addition, administration of citalopram or venlafaxine may be recommended in patients treated with digoxin because an in vitro study showed that inhibitory effects of these drugs on P-glycoprotein are weak.[4]

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# Identification of a Single Time-point for Plasma Lansoprazole Measurement That Adequately Reflects Area Under the Concentration-Time Curve

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**Abstract:** The objective of this study was to identify a single time-point for plasma lansoprazole measurement that adequately reflects area under the plasma lansoprazole concentration-time curve (AUC) after administration of lansoprazole alone or together with coadministration with CYP mediators. A randomized double-blind placebo-controlled crossover study design in 3 phases was conducted at intervals of 2 weeks. Eighteen healthy Japanese volunteers, comprising 3 CYP2C19 genotype groups, took a single oral 60-mg dose of lansoprazole after three 6-day pretreatments, that is, clarithromycin 800 mg/d, fluvoxamine 50 mg/d, and placebo. Blood samplings (10 mL each) for determination of lansoprazole were taken up to 24 hours after the administration of lansoprazole. Correlation between plasma lansoprazole concentrations at various time points and $AUC_{0-24}$ were analyzed. Although there were significant differences in the pharmacokinetic parameters of lansoprazole during clarithromycin and placebo among CYP2C19 genotypes, the differences were not found during fluvoxamine. The plasma concentrations 3, 4, 6, and 8 hours after administration (C3, C4, C6, and C8, respectively) were highly correlated with $AUC_{0-24}$ in coadministration with placebo, clarithromycin, and fluvoxamine ($r > 0.8$, $P < 0.001$). In particular, C6 showed a correlation coefficient of 0.940, 0.992, and 0.953 in coadministration with placebo, clarithromycin, and fluvoxamine, respectively, and was the most appropriate for estimating $AUC_{0-24}$. The present study demonstrates that AUC of lansoprazole can be estimated by using a single time-point at C6. This method of plasma concentration monitoring at one time-point might be more suitable for AUC estimation than reference to CYP2C19 genotypes, particularly in coadministration of CYP mediators.

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**Key Words:** lansoprazole, AUC, single time-point, CYP2C19 genotype, phenotype

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Lansoprazole is a proton pump inhibitor (PPI) that suppresses gastric acid secretion by inhibiting $H^+$, $K^+$-ATPase in the secretory membrane of gastric parietal cells.[1] Lansoprazole is effective in the treatment of various peptic diseases, including gastric and duodenal ulcer, reflux esophagitis, and Zollinger-Ellison syndrome.[2]

Lansoprazole is inactivated by metabolism in the liver, especially by CYP2C19 and CYP3A4 of the cytochrome P450 family, and differences in the metabolic capacities of subjects could have a profound influence on drug efficacy.[3] CYP2C19 shows genetically determined polymorphism, yielding extensive metabolizers (EMs) and poor metabolizers (PMs).[4] Several in vivo studies have consistently suggested that the area under the plasma lansoprazole concentration-time curve (AUC) in CYP2C19 PMs is much greater than that in EMs.[5,6] Therefore, CYP2C19 plays an important role in lansoprazole metabolism in EMs, whereas sulfoxidation of lansoprazole, catalyzed by CYP3A4, could be the predominant metabolic pathway in PMs.

The acid suppressive effect of PPIs such as lansoprazole is reported to be correlated with AUC.[7–9] It is also reported that CYP2C19 genotyping is a useful tool for guiding *Helicobacter pylori* eradication, and treatment effect in patients with reflux esophagitis.[3] On the other hand, we showed that the significant difference in AUC of omeprazole between EMs and PMs was not observed when fluvoxamine, which was reported to inhibit CYP2C19 activity,[10,11] was combined with lansoprazole, suggesting that lansoprazole AUC could not be estimated based on the difference in genotypes between EMs and PMs.[12] Therefore, although it is evident that CYP2C19 genotypes are indicators for estimating AUC of lansoprazole, it is necessary to recognize that the estimation should be performed by using both genotype and measured AUC value in individuals in some clinical settings. However, many blood collection time points are required to calculate AUC accurately. Taking account of the time required for blood collection, the burden on

patients and the cost to measure plasma concentrations, AUC measurement is not appropriate for therapeutic drug monitoring of the PPIs.

The purpose of this study is to identify a single time-point to measure a plasma lansoprazole concentration that adequately reflects AUC in a single administration of lansoprazole and in coadministration with clarithromycin, an inhibitor of CYP3A4, and with fluvoxamine, an inhibitor of CYP2C19.

## METHODS

### Study Design

Eighteen healthy Japanese volunteers (9 men and 9 women) who were *H. pylori*-negative were enrolled in this study. Their mean age was 25.1 ± 3.8 years and mean body weight was 56.6 ± 13.3 kg. Most subjects in this study had participated in our previous studies.[12,13] The Ethics Committee of Hirosaki University School of Medicine approved this study protocol, and written informed consent was obtained from each participant before any examinations. The mutated alleles for CYP2C19, CYP2C19*3(*3), and CYP2C19*2(*2) had been identified using polymerase chain reaction-restriction fragment length polymorphism methods of De Morais et al,[14] before this study. The CYP2C19 genotype analyses revealed 5 different patterns as follows: *1/*1 in 6, *1/*2 in 3, *1/*3 in 3, *2/*2 in 5, and *2/*3 in 1. These were divided into 3 groups, homozygous EMs (*1/*1, $n = 6$), heterozygous EMs (*1/*2 and *1/*3, $n = 6$), and PMs (*2/*2 and *2/*3, $n = 6$).

A randomized double-blind placebo-controlled crossover study design in 3 phases was conducted at intervals of 2 weeks. Clarithromycin (400 mg) as a capsule containing the equivalent of 2 tablets (Clarith, Taisho Pharmaceutical Co, Ltd, Tokyo, Japan), fluvoxamine (25 mg) as a capsule containing the equivalent of a tablet (Luvox, Fujisawa Pharmaceutical Co, Ltd, Osaka, Japan), or matched placebo was given orally twice a day (9 AM, 9 PM) for 6 days. Six volunteers within each group were allocated to each of the 3 different drug sequences: placebo-clarithromycin-fluvoxamine, fluvoxamine-placebo-clarithromycin, or clarithromycin-fluvoxamine-placebo. On day 6, they took a single oral 60-mg dose of lansoprazole (Takepron, Takeda Pharmaceutical Co, Ltd, Osaka, Japan) with 400 mg dose of clarithromycin, 25 mg dose of fluvoxamine, or placebo after overnight fasting (9 AM). No other medications were taken during the study periods. No meal was allowed until 4 hours after the dosing (1 PM). The use of alcohol, tea, coffee, and cola was forbidden during the test days.

### Blood Samplings

Blood sampling (10 mL each) for determination of lansoprazole were taken into heparinized tubes just before and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 hours after the administration of lansoprazole. Plasma was separated immediately and kept at −30°C until analysis.

### Assay

Plasma concentrations of lansoprazole were quantified using high-performance liquid chromatography (HPLC) method developed in our laboratory.[15] In brief, after alkalization with 0.5 mL NaOH (0.001 mol/L), 1 mL plasma was extracted with 5 mL of diethyl ether-dichloromethane (70:30, vol/vol). The organic phase was evaporated to dryness at 60°C. Samples dissolved into mobile phase of phosphate buffer (0.02 mol/L, pH = 4.6), acetonitrile, and methanol (55:40:5, vol/vol) were injected onto a C18 STR ODS-II HPLC analytical column (column II; 150 × 4.6 mm ID, particle size 5 ím; Shinwa Chemical Industry, Kyoto, Japan) connected to a SHIMADZU CLASS-VP, SHIMADZU Corporation, Kyoto, Japan, HPLC system. The flow rate was 0.8 mL/min and the wavelength was set at 286 nm. The limit of quantification was 3 ng/mL for lansoprazole. Intraday and interday coefficient of variability were less than 6.1% and 5.1% for lansoprazole, respectively, at the lowest concentration ranges.

### Pharmacokinetic Data Analyses

The peak concentration ($C_{max}$) and time to peak concentration ($t_{max}$) were obtained directly from the original data. $AUC_{0-24}$ was calculated using the trapezoidal rule. The terminal rate constant ($k_e$) used for the extrapolation was determined by regression analysis of the log-linear part of the concentration-time curve for each subject. The elimination half-life was determined from $0.693/k_e$.

### Statistical Analyses

One-way analysis of variance and Fisher exact tests were used for comparisons among the 3 CYP2C19 genotypes and clinical profiles such as age, body weight, and sex. The 2-way analysis of variance for the comparison of placebo versus either clarithromycin or fluvoxamine was conducted on pharmacokinetic parameters, although they were analyzed for statistical differences using Bonferroni correction. Correlation between lansoprazole concentrations at various time points and $AUC_{0-24}$ was tested using Spearman rank test. A $P$ value of 0.05 or less was regarded as significant. SPSS 12.0J for Windows (SPSS Japan Inc, Tokyo) was used for these statistical analyses.

Predicted $AUC_{0-24}$ using a linear regression model was compared with observed $AUC_{0-24}$ by calculating the mean-squared error (MSE) for precision, using the following equation.[16]

$$MSE = \frac{\sum_{i=1}^{n}(PE)^2}{n} \quad (1)$$

Precision assessments were compared by calculating ΔMSE, where it was calculated using the following equations.[16]

$$\Delta MSE = \frac{\sum_{i=1}^{n}(PE^2 \text{ of C3, 4 or 8} - PE^2 \text{ of C6}}{n} \quad (2)$$

**TABLE 1.** Pharmacokinetic Parameters of Lansoprazole During Control, Clarithromycin Treatment, and Fluvoxamine Treatment in Homozygous-EMs, Heterozygous-EMs, and PMs for CYP2C19

| Pharmacokinetic Parameters | Treatments | Homozygous-EMs (n = 6) | Heterozygous-EMs (n = 6) | PMs (n = 6) |
|---|---|---|---|---|
| $C_{max}$ (ng/mL) | With placebo | 2685 ± 971 | 2924 ± 1385 | 4231 ± 1519 |
| | With clarithromycin | 3544 ± 1747 | 4734 ± 2098* | 5997 ± 1136** |
| | With fluvoxamine | 4125 ± 1277 | 3549 ± 2036 | 3746 ± 1837 |
| $t_{max}$ (h) | With placebo | 1.75 ± 0.69 | 2.08 ± 0.80 | 2.25 ± 0.61 |
| | With clarithromycin | 1.58 ± 0.20 | 1.67 ± 0.75 | 1.58 ± 0.38 |
| | With fluvoxamine | 2.08 ± 0.80 | 2.42 ± 0.92 | 3.83 ± 2.23 |
| Elimination half-life (h) | With placebo | 0.98 ± 0.35 | 1.69 ± 0.51 | 3.69 ± 1.40 |
| | With clarithromycin | 1.32 ± 0.56 | 1.93 ± 0.50 | 6.01 ± 1.54** |
| | With fluvoxamine | 2.90 ± 1.09** | 2.84 ± 0.66* | 4.87 ± 0.66 |
| $AUC_{0-24}$ (ng/mL/h) | With placebo | 6373 ± 3446 | 8906 ± 3523 | 26666 ± 8058 |
| | With clarithromycin | 10329 ± 8748 | 15273 ± 6836* | 50295 ± 17588** |
| | With fluvoxamine | 24390 ± 10022** | 22237 ± 13135* | 27774 ± 11818 |

Data are shown as mean ± SD.
*$P < 0.05$, **$P < 0.01$, compared with placebo.

Prediction error (PE) = predicted ACU − observed ACU. The 95% confidence interval (CI) was calculated on ΔMSE and the ΔMSE was regarded as not precise when the higher 95% CI did not exceed 0.

## RESULTS

None of the subjects needed to be withdrawn from this study. No differences between the CYP2C19 genotypes, homozygous EMs, heterozygous EMs, and PMs were found in subject demographics, including age (mean ± SD, 25 ± 4, 25 ± 5, and 26 ± 4 y;NS), body weight (57 ± 16, 53 ± 10, and 60 ± 15 kg; NS), and sex (M/F; 3/3, 3/3, and 3/3; NS).

The pharmacokinetic parameters of lansoprazole during 3 phases in each CYP2C19 genotype group are summarized in Table 1. During placebo administration, there were significant differences in $AUC_{0-24}$ ($P < 0.001$) and elimination half-life ($P < 0.001$) of lansoprazole among CYP2C19 genotypes. Clarithromycin treatment significantly increased $C_{max}$ and $AUC_{0-24}$ of lansoprazole in heterozygous EMs ($P < 0.05$) and in PMs ($P < 0.01$), and prolonged elimination half-life by 1.6-fold ($P < 0.05$) in PMs. Fluvoxamine pretreatment significantly increased $AUC_{0-24}$ of lansoprazole by 3.8-fold in homozygous EMs ($P < 0.01$) and 2.5-fold in heterozygous EMs ($P < 0.05$), and prolonged elimination half-life by 3.0-fold in homozygous EMs ($P < 0.01$) and by 1.7-fold in heterozygous EMs ($P < 0.05$), respectively. There were no differences in $AUC_{0-24}$ during fluvoxamine treatment among CYP2C19 genotypes.

Regarding the correlation of plasma lansoprazole concentrations at various time points with $AUC_{0-24}$, the plasma concentrations 3, 4, 6, and 8 hours after administration (C3, C4, C6, and C8, respectively) showed high correlation coefficients ($r$) (not less than 0.8, $P < 0.001$) in coadministration with placebo, clarithromycin, and fluvoxamine (Table 2). The time point C6 showed a particularly good correlation coefficient of 0.940, 0.992, and 0.953 in coadministration with placebo, clarithromycin, and fluvoxamine, respectively, and was the most appropriate for estimating $AUC_{0-24}$. The plasma concentrations beyond 12 hours after administration were below the quantification limit in several subjects, decreasing the correlation coefficient.

The C6 value showed the highest $r^2$ and the least MSE in the linear regression analysis of plasma lansoprazole concentrations at various time points with $AUC_{0-24}$. The higher 95% CI of all ΔMSE with C6 exceeded 0 when compared with C3, C4, and C8,

**TABLE 2.** Correlation Between Plasma Lansoprazole Concentrations at Various Time Points and $AUC_{0-24}$ in Coadministration With Placebo, Clarithromycin, and Fluvoxamine

| | With Placebo (n = 18) | | With Clarithromycin (n = 18) | | With Fluvoxamine (n = 18) | |
|---|---|---|---|---|---|---|
| Sampling Time (h) | *r* | *P* | *r* | *P* | *r* | *P* |
| 0.5 | 0.293 | 0.239 | 0.140 | 0.579 | −0.415 | 0.087 |
| 1 | 0.375 | 0.126 | 0.313 | 0.206 | −0.086 | 0.735 |
| 1.5 | 0.564 | 0.015 | 0.899 | < 0.001 | 0.063 | 0.804 |
| 2 | 0.694 | < 0.001 | 0.893 | < 0.001 | 0.628 | 0.005 |
| 3 | 0.889 | < 0.001 | 0.915 | < 0.001 | 0.884 | < 0.001 |
| 4 | 0.934 | < 0.001 | 0.973 | < 0.001 | 0.948 | < 0.001 |
| 6 | 0.940 | < 0.001 | 0.992 | < 0.001 | 0.953 | < 0.001 |
| 8 | 0.909 | < 0.001 | 0.979 | < 0.001 | 0.905 | < 0.001 |
| 12 | 0.783 | < 0.001 | 0.940 | < 0.001 | 0.853 | < 0.001 |
| 24 | 0.514 | 0.029 | 0.705 | < 0.001 | 0.471 | 0.049 |

suggesting that probability at C6 is not less than other time points. Prediction precision of C6 was the best even in the subject whose $t_{max}$ was the longest ($t_{max}$ = 8 h).

## DISCUSSION

CYP2C19 genotype is regarded as a useful tool to predict the pharmacokinetics of lansoprazole because of the major contribution of CYP2C19 to lansoprazole metabolism.[5,6] However, it has been reported that CYP2C19 activity decreases in patients with hepatic dysfunction,[17] and that the AUC of lansoprazole increases more in patients with hepatic dysfunction[18] or in the elderly[19] than in young healthy subjects. The present study revealed that fluvoxamine coadministration masked the effects of CYP2C19 genotypes on lansoprazole kinetics. Therefore, identification of CYP2C19 genotype alone was sometimes insufficient to estimate AUC of lansoprazole in cases with decreased CYP2C19 activity or in the setting of such a drug-drug interaction.

Furuta et al[20] have already reported that not only CYP2C19 but also C3 is a useful indicator for AUC and, hence, treatment effect in reflux esophagitis patients. However, no study has investigated the exact time-point after lansoprazole administration to measure a plasma concentration which correlates with AUC. The result of the present study showed favorable correlations between $AUC_{0-24}$ and plasma lansoprazole concentrations of C3, C4, C6, and C8, regardless of whether there were coadministered drugs (eg, clarithromycin or fluvoxamine). Prediction precision of $AUC_{0-24}$ was suggested by the least prediction errors associated with the above time points. Taking into account the correlation coefficients and prediction precisions, it is reasonable to conclude that C6 was the best time point to estimate AUC.

Clarithromycin, an inhibitor of CYP3A4[21,22] is concomitantly administrated with lansoprazole in *H. pylori* eradication. Several in vivo studies have suggested that clarithromycin coadministration increases the plasma PPIs such as omeprazole concentration.[23–25] It is likely that the drug interaction between clarithromycin and lansoprazole through CYP3A4 and possibly P-glycoprotein inhibition as reported by Saito et al[13] occurred in this study. Our results suggest that C6 is the best time-point to estimate AUC of lansoprazole, even when clarithromycin is administered in *H. pylori* eradication.

This study has the limitations of a single-dose kinetic study. Although there is no information indicating any differences in pharmacokinetics between single-dose and repeated-doses of lansoprazole, it has been reported that repeated-doses of omeprazole induced the metabolism of omeprazole.[6] Further studies are required to confirm whether or not plasma concentration monitoring of lansoprazole at one time point is clinically and pharmacoeconomically relevant.

In conclusion, the present study demonstrated that AUC of lansoprazole can be estimated by one defined sampling time point of C6. This method of plasma concentration monitoring might be more suitable for AUC estimation than reference to CYP2C19 genotype alone, particularly when the drug is coadministered with CYP mediators. However, further studies are required to confirm whether plasma concentration monitoring at one time point is clinically relevant.

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ORIGINAL INVESTIGATION

# ROCK inhibition produces anxiety-related behaviors in mice

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**Abstract**
*Rationale* The role of Rho/Rho-associated kinase (ROCK) in regulating dendritic and axonal morphology during development has gained much attention. Very little is known, however, about the role of the Rho/ROCK pathway in emotional behavior.
*Objective* To investigate the role of ROCK in emotional behaviors. We examined how the ROCK inhibitor Y27632 affects the performance of mice on three behavioral tests that measure anxiety-related behaviors.
*Results* In the elevated plus-maze test, Y27632 (10 nmol, intracerebroventricular) induced a significant decrease in the percentage of time spent in the open arms and in the percentage of entries into open arms. In the fear conditioning test, Y27632-treated mice froze significantly more often and longer than did saline-treated mice. In the hole-board test, Y27632 significantly suppressed head-dipping behavior in Y27632-treated mice than in saline-treated mice. On the other hand, Y27632 did not produce on spontaneous alteration performance in the Y-maze test. These results indicate that ROCK inhibition increased anxiety-related behaviors.
*Conclusion* Our findings suggest that the ROCK pathway is involved in the expression of anxiety- and fear-related behaviors. Furthermore, we propose that if the Rho/ROCK pathway plays an important role in mediating anxiety-related behaviors in humans, it may prove to be a novel system for anxiolytics to target.

**Keywords** Y27632 · ROCK inhibitor · Rho/ROCK pathway · Elevated plus maze · Fear conditioning · Anxiety

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## Introduction

The low-molecular-mass GTPases of the Rho subfamily (RhoA, B, C, G, H, Rac1, Rac2, and Cdc42) are implicated in a variety of biological responses, including cell motility, gene expression, and cell-cycle progression (Tapon and Hall 1997; Hall 1998; Kaibuchi et al. 1999). Rho family proteins, like other GTP-binding proteins, serve as molecular switches that cycle between GDP-bound inactive and GTP-bound active states. Upon binding to GTP, Rho activates downstream effectors such as Rho-associated protein kinase (ROCK) and phosphatidylinositol 4-phosphate 5-kinase (Machesky and Hall 1996; Narumiya 1996; Ishizaki et al. 1997). Among these effectors, ROCK plays an important role in generating contractile force by increasing myosin light chain phosphorylation (Amano et al. 1996; Kimura et al. 1996). Rho regulates cofilin via ROCK and LIM kinases; this signal transduction pathway modulates actin assembly in many cell types in response to various extracellular stimuli (Maekawa et al. 1999). A growing body of evidence indicates that the Rho/ROCK

pathway is involved in intracellular membrane trafficking such as exocytosis, endocytosis, and phagocytosis (Lamaze et al. 1996; Caron and Hall 1998; Yamaguchi et al. 2000). The Rho/ROCK pathway has also been implicated in regulating dendritic and axonal morphology during development (Van Aelst and Cline 2004). Furthermore, inhibition of the Rho/ROCK pathway has been shown to effectively reduce the pathology and symptomology observed in several in vivo animal models of stroke, inflammatory and demyelinating diseases, Alzheimer's disease, and neuropathic pain (see review by Mueller et al. 2005). Tanaka and colleagues recently reported that ROCK inhibitor *trans*-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide (Y-27632), which selectively inhibits the activities of two isoforms of ROCK (ROCK-I and ROCK-II) and suppresses RhoA-mediated cell transformation (Davies et al. 2000), produced functional recovery after spinal cord injury in 12-week-old rats (Tanaka et al. 2004). These results suggest that the ROCK pathway plays an important role in adulthood as well as in development. Recently, the kind of regulation of dendritic and axonal morphology that occurs during development has also been proposed to be involved in the expression of pharmacological actions induced by anxiolytics and antidepressants (Magarinos et al. 1999; Norrholm and Ouimet 2000, 2001; Hajszan et al. 2005). In addition, we found the decreased expression of several Rho/ROCK pathway components in the frontal cortex of rats 24 h after a single administration of several different antidepressants [selective serotonin (5-HT) reuptake inhibitors, 5-HT/norepinephrine reuptake inhibitors, and tricyclic antidepressants] when compared to the gene expression in rat frontal cortex after acute treatment with antidepressants using a differential cloning strategy (Yamada et al. 2000–2002; Yamada and Higuchi 2002; Nishioka et al. 2003; Takahashi et al. 2005). Very little is known, however, about the role of the Rho/ROCK pathway in emotional behaviors.

The changes in emotional behavior have been studied in several animal models. The elevated plus-maze test has become a popular animal model in the field of anxiety, and the results are sensitive to benzodiazepine anxiolytic treatment (Pellow et al. 1985). The hole-board test provides a simple method for measuring the response of an animal to an unfamiliar environment (Boissier and Simon 1962). Previously, the hole-board test has been used to assess the anxiety or responses to stress in mice (Rodriguez Echandia et al. 1987; Tsuji et al. 2000). In fear conditioning, a neutral conditioned stimulus (CS), such as a tone, acquires the capacity to elicit defensive responses after being associated with an aversive unconditioned stimulus, typically a footshock. It has been recognized that this model might be a stress model reflecting the fear memory (Fanselow 1980; Fanselow and Tighe 1988; LeDoux 1995). The Y-maze test assesses spontaneous alteration behavior, a natural tendency in mice to explore less recently visited arms in the maze. This behavior is thought to be based on spatial working memory and/or cognition (Warburton and Heise 1972; Sarter et al. 1988) because drugs producing amnesia, such as scopolamine and dizocilpine, have been shown to impair performance on the Y-maze (Sarter et al. 1988; Parada-Turska and Turski 1990; Stone et al. 1991). Thus, to investigate the role of ROCK in emotional behaviors, we examined the effects of Y27632 on the performance of mice on the elevated plus-maze test, hole-board test (an anxiety test that does not rely on memory), the auditory fear conditioning test (a fear memory test that relies on memory), and on the Y-maze test (a nonanxiety test that assesses short-term memory and cognition).

## Materials and methods

### Animals

Male ICR mice were used for the behavioral experiments (age: 5–6 weeks, Tokyo Laboratory Animals Science, Tokyo, Japan). They were housed in groups (ten mice per cage) in a temperature-controlled environment with a 12-h light/12-h dark cycle and were given free access to food and water. Different mice were used for each test. All studies were carried out in accordance with the Declaration of Helsinki and the Institutional Guide for the Care and Use of Laboratory Animals accredited by the Ministry of Education, Science, Sports, and Culture of Japan.

### Intracerebroventricular injection

Twenty-four hours before intracerebroventricular (i.c.v.) drug or vehicle injections, mice were anesthetized with diethyl ether. A 3-mm barrel housing two needles (tip: 27-gauge × 3 mm and base: 22-gauge × 5 mm; Natume Seisakusho, Tokyo, Japan) attached to a 25-μl Hamilton microsyringe was positioned 2 mm from either side of the midline, between the anterior roots of the ears, and was used to mark the position of burr holes for the unilateral injection. The barrel assembly was glued in place with dental cement. On the day of the i.c.v. injection, the mouse's head was fixed to a V-shaped holder, and the barrel housing the needles was lowered 3.0 mm ventral to the brain surface into the lateral ventricle. The vehicle or drugs (5 μl per mouse) were then injected into the ventricle through the Hamilton microsyringe according to the method of Kamei et al. (2004). No anesthetics were used during the actual injection. After each experiment, the position of the injection sites was marked by injecting dye solution through the syringe.

### Elevated plus-maze testing

The plus maze (Neuroscience, Tokyo, Japan) consisted of a black Plexiglas apparatus with two open arms (5×65 cm) and two enclosed arms (5×65×40 cm). Mice were moved to the testing room (30 lx) at 9:00 a.m. to habituate them to the experimental environment. The entire apparatus was located in a separate room (4 lx). The maze was elevated 41 cm above the floor and was placed in indirect light. The animals were injected with either drug or vehicle (see below) and tested 30 min later. At the beginning of the 5-min test session, each mouse was placed in the central neutral zone, facing one of the open arms. The total number of visits to the closed and open arms and the cumulative time spent in and the visits into the open arms were then determined automatically on a monitor through a CCD video camera system. An arm visit was recorded when the mouse placed all four paws into the arm. The total locomotor activity (in centimeters) was assessed by the total distance the mice traveled in mice performing an elevated plus-maze test. All data were stored in a personal computer and were analyzed with analytical motion software (Video Image Motion Analysis Program, Axis 60 Plus Maze; Neuroscience, Tokyo, Japan). The ratio between cumulative time spent or visits in the open arms and the cumulative time spent or visits in total arms was expressed as percentage time spent in the open arms or open arm entry. A computer system recorded the time spent and the number of entries in the open or closed arms by means of infrared photocells, which were mounted 5 cm proximal to each arm, to make sure that the mice entered that arm completely (Ikeda et al. 2006). The apparatus was wiped clean with water and dried after each subject. The elevated plus-maze tests were conducted between 11:00 a.m. and 1:00 p.m.

### Auditory fear conditioning and testing

Fear conditioning of mice took place in a brightly lit Plexiglas rodent conditioning chamber with a metal grid floor (ENV-307A; MED Associates, VT, USA). The floor of the chamber consisted of 24 stainless steel rods (diameter, 3 mm) spaced 5 mm apart (center-to-center), which were connected to a shock generator (Dual Range Constant Current Shock Source ENV-410; MED Associates, VT, USA). The chamber was placed into a soundproof box (60×50×50 cm; NSB-B101; Neuroscience, Tokyo, Japan). During testing, the context of the chamber was altered by dimming the light. Mice were moved from the testing room at 9:00 a.m. to habituate them to the experimental environment; training started at 1:00 p.m. One group of mice received training 30 min after injections, whereas the other group of mice received injections immediately after training. Training consisted of five conditioning trials, which were five pairings of a 10-s tone CS (3 kHz, 85±1 dB) that coterminated with a footshock unconditioned stimulus (UC) (1 s, 1.5 mA). The mice were allowed to freely explore the apparatus in the pretone period (Pre-CS) during the 360-s period before presentation of the first CS. After the Pre-CS, the mice were exposed to the CS during the 240-s period of auditory fear conditioning. The total training session was performed for 600 s. The delivery of the footshock was controlled by software (Freeze Frame-4; Actimetrics, IL, USA). The intertrial interval varied randomly between 10 and 50 s. Drugs were injected i.c.v. 30 min before (pretraining treatment) or immediately after (posttraining treatment) the training session. After the conditioning, mice were returned to a single cage (10×15×13 cm). This training protocol represents a modification of the method of Burghardt et al. (2004). The footshock intensities used in the present study were determined as described by Saitoh et al. (2004). We selected these protocols because our pilot studies revealed that they produce, on average, about 30% of the maximal level of freezing in control animals. Thus, with this protocol, ceiling effects were avoided, allowing us to detect anxiogenic (e.g., increased freezing) effects of the treatment. Mice were tested drug-free 24 h after they were conditioned. Eleven presentations of the 10-s tone CS (intertrial interval, 20 s) were given within 360 s. A video camera mounted on top of the testing chamber recorded behavior during training and testing sessions for later scoring. Fear-related behavior was evaluated by tracking the number of seconds mice froze during each tone presentation. We defined "freezing" as the lack of all movements with the exception of respiration. The conditioned fear tests were conducted between 1:00 and 5:00 p.m. All data were stored in a personal computer and were analyzed with analytical software (Freeze Frame-4; Actimetrics, IL, USA).

### Hole-board testing

Performance in the hole-board test (Boissier and Simon 1962) was assessed with an automated system as described previously (Takeda et al. 1998). The hole-board apparatus was made of a gray wooden box (50×50×50 cm) having four holes, 3 cm in diameter, equally spaced in the floor. An infrared sensor beam attached to one of the box's wall detected the number and duration of rearing and head-dipping behaviors and the latency to the first head-dipping behavior. Other behavioral parameters, such as the location and movement distance [total locomotor activity (in centimeters)] of mice, were recorded by an overhead color CCD camera. We painted the heads of the mice yellow to facilitate tracking of the mice with the color CCD camera, and the color CCD camera followed their center of gravity. Data from the CCD camera were collected as a time sequence of reflection signals

through a custom-designed interface (CAT-10, Muromachi Kikai, Japan). Head-dipping behaviors were double-checked by comparing sensor readings obtained from the infrared sensor beam with movie images of the mice taken with the overhead color CCD camera. Thus, only when both the head intercepted the infrared beam and the head was detected at the hole by the CCD camera was head-dipping behavior counted. All of the data were analyzed and stored in a personal computer using analytical software (Comp ACT HBS, Muromachi Kikai, Japan). The animals were restrained in a plastic snug-fit apparatus (3 cm in diameter and 7 cm in length) (stressed group) or left in their home cage (non-stressed group). Thirty minutes later, mice were exposed to acute restraint stress for 30 min, and then the exploratory behavior was measured for 5 min on the hole board (stress group). The exploratory behavior in nonstressed mice group was measured 60 min after administration. Each animal was injected with drugs or vehicle 30 min before being subjected to the stressful restraints. For testing, each animal was placed in the center of the hole board and allowed to freely explore the apparatus for 5 min. Total locomotor activity, number of rearing and head-dipping behaviors, and latency of the first head-dipping behavior were recorded automatically. This procedure was based on that described by Tsuji et al. (2001).

### Y-maze testing

A black-painted Y-maze made of plywood was used for these experiments. Each arm was 40 cm long and had 12-cm-high walls; the walls were 3 cm wide at the bottom and 10 cm wide at the top. The arms of the Y-maze were positioned at equal angles. The testing procedure was based upon that of Sarter et al. (1988). Each mouse was placed at the end of one arm and was allowed to move freely through the maze for an 8-min test session. The arm visits of maze were counted and recorded when the mouse placed all four paws into the arm by the observer. The experiments were blind with respect to the drug treatment. After the testing period, we calculated the number of maximum alternations for each mouse, which equaled the total number of times a mouse entered the arms minus 2. We also calculated the percent alternation according to the following formula: (actual alternations/maximum alternations) × 100. For example, if the three arms are designated A, B, and C, and a mouse consecutively enters the arms according to the sequence ACBABACBAB, the mouse's performance would consist of five alternations (ACB, CBA, BAC, ACB, and CBA) out of eight (10 minus 2) possible alternations (62.5%). The results in percent arms less than eight times during the test were not used because the data obtained from these mice were not considered to reflect precise alternation. Two groups of mice were injected whether with vehicle or Y27632 (10 nmol). The exploratory behaviors of mice measured 30 min after administration.

### Drugs

The ROCK inhibitor *trans*-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide (Y27632; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was dissolved in physiological saline. The prototypical benzodiazepine anxiolytic, diazepam (Cercine®, Takeda Chemical Industries, Osaka, Japan), which was an injection dissolved in 40% benzyl alcohol, 10% ethanol, 1.5% propylene glycol, and 42.8 mg/ml benzoic acid, used Cercine® diluted with 0.1% Tween 80 physiological saline at the time of preparation. The i.c.v. doses of Y27632 were based on previous reports (Zhou et al. 2003; Inoue et al. 2004).

### Data analysis

The data are expressed as means±S.E. The statistical significances of differences in the elevated plus-maze test and auditory fear conditioning test between Y27632 treatment groups were assessed with one-way analysis of variance (ANOVA), and post hoc individual group comparisons were made with Bonferroni/Dunn's test. The statistical significances of differences in hole-board test data between Y27632 treatment groups were assessed with two-way analysis of variance (ANOVA; stress × drug manipulation), and post hoc individual group comparisons were made with Bonferroni/Dunn's test. The statistical significances of differences between diazepam treatment groups were assessed with a Student's *t* test. Analyses were made using StatView 5.0 statistical software (SAS Institute, Cary, NC, USA). *P*-values of less than 0.05 were considered significant.

## Results

### Effects of ROCK inhibition on anxiety-related behaviors as assessed in the elevated plus maze

As shown in Fig. 1a, administration of diazepam (60 nmol, i.c.v.), a typical benzodiazepine anxiolytic, produced a significant increase in the percentage of time spent in the open arms ($P<0.05$) and an increase in the percentage of entries into open arms compared with vehicle treatment. Administration of Y27632 (i.c.v; 10 nmol) produced a significant decrease in the percentage of time spent in the open arms [$F(3,41)=5.156$, $P<0.05$] and the percentage of entries into open arms [$F(3,41)=5.423$, $P<0.05$] (Fig. 1a). In contrast, Y27632 and diazepam failed to produce significant effects on locomotor activity, as assessed by the number of total arm entries over the range of doses

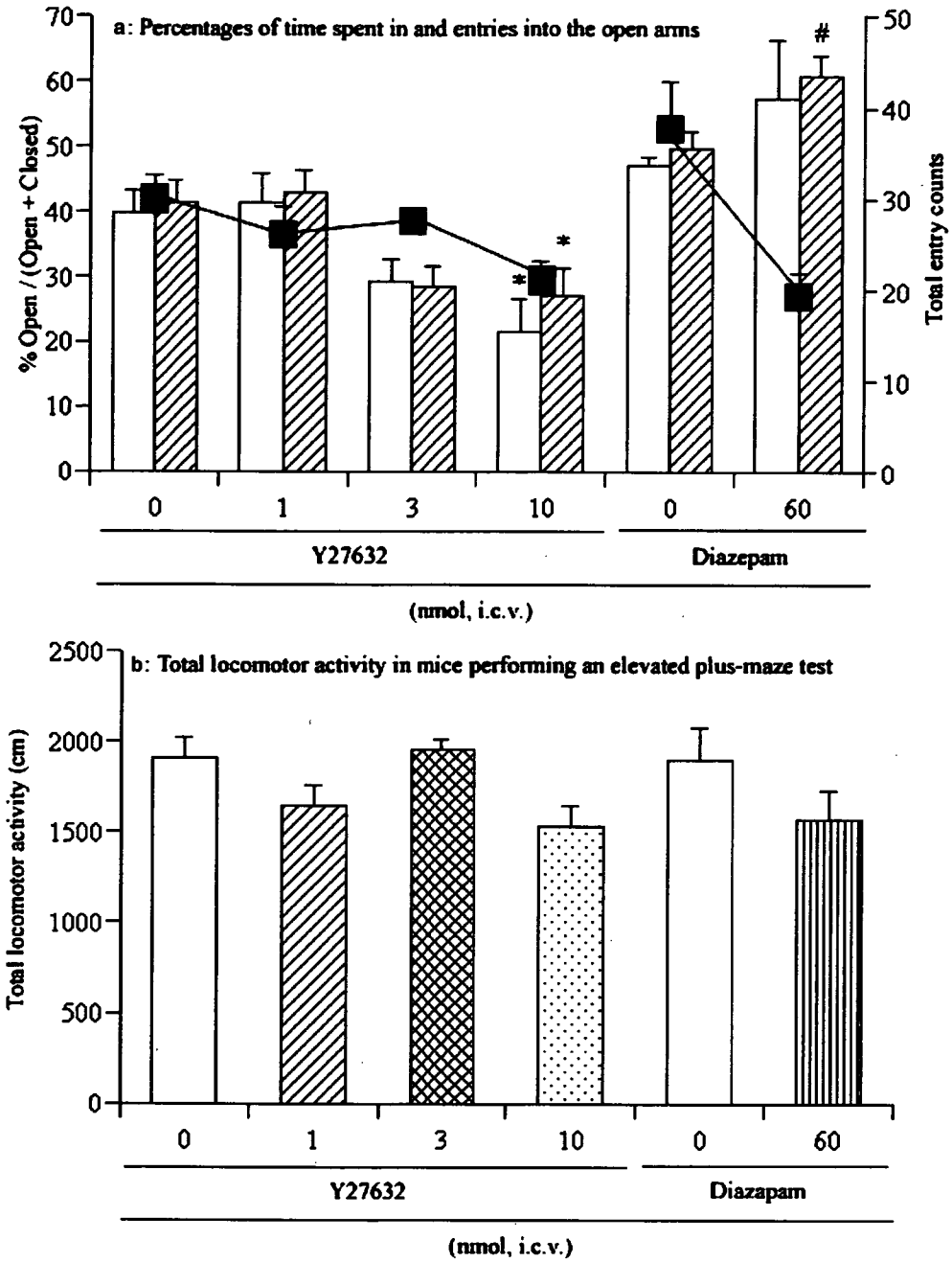



**Fig. 1** Effect of ROCK inhibition on anxiety-related behaviors in the elevated plus-maze test. *Panel* **a** shows the percentages of time spent (*open column*) in and entries (*shaded column*) into the open arms in mice placed for 5 min in the elevated plus maze (*Y-axis, left*). *Closed square* represents the locomotor activity, as assessed by the number of total arm entries (*Y-axis, right*). *Panel* **b** shows the total locomotor activity (in centimeters), as assessed by the total distance the mice traveled in mice performing an elevated plus-maze test. The data are expressed as means± S.E. The statistical significances of differences between Y27632 treatment groups were assessed with one-way analysis of variance (ANOVA), and post hoc individual group comparisons were made with Bonferroni/Dunn's test. The statistical significances of differences between diazepam treatment groups were assessed with a Student's *t* test. [Y27632 (saline, $n$=15; 1 nmol, $n$=11; 3 nmol, $n$=9; 10 nmol, $n$=10) or diazepam (vehicle, $n$=6, 60 nmol, $n$=6)]. *$P$<0.05 vs saline-treated mice (Y27632 treatment groups), #$P$<0.05 vs vehicle-treated mice (diazepam treatment groups)

tested (Fig. 1a). In addition, Y27632 and diazepam did not affect exploratory behavior, as assessed by the total distance the mice traveled (i.e., total locomotor activity), at all doses tested (Fig. 1b).

## Effects of ROCK inhibition on the acquisition of auditory fear conditioning

To evaluate anxiety-related behaviors in a different test using our animal model, we employed a fear conditioning paradigm that measures fear-associative learning in mice. The percent freezing displayed by mice pretreated with either drug or vehicle ($n$=6–9) in response to an auditory fear-CS is shown in Fig. 2b. The Pre-CS (pretone period) represents the general motor activity of the mice during the 20-s period before presentation of the first CS, and the CS represents the freezing behavior of the mice during the 240-s period of auditory fear conditioning. Administration (i.c.v.) of either drug or saline did not significantly affect the freezing behavior of mice during the pretone period (Pre-CS) or during the CS and posttone period. The following day, when mice were tested drug-free to presentation of the tone alone (Fig. 2a), on average, Y27632-treated mice froze significantly more often and longer than did saline-treated mice during the presentation of all 11 tones [$F$(3,50)=4.099, $P$<0.05) (Fig. 2a). The average responses during individual trials

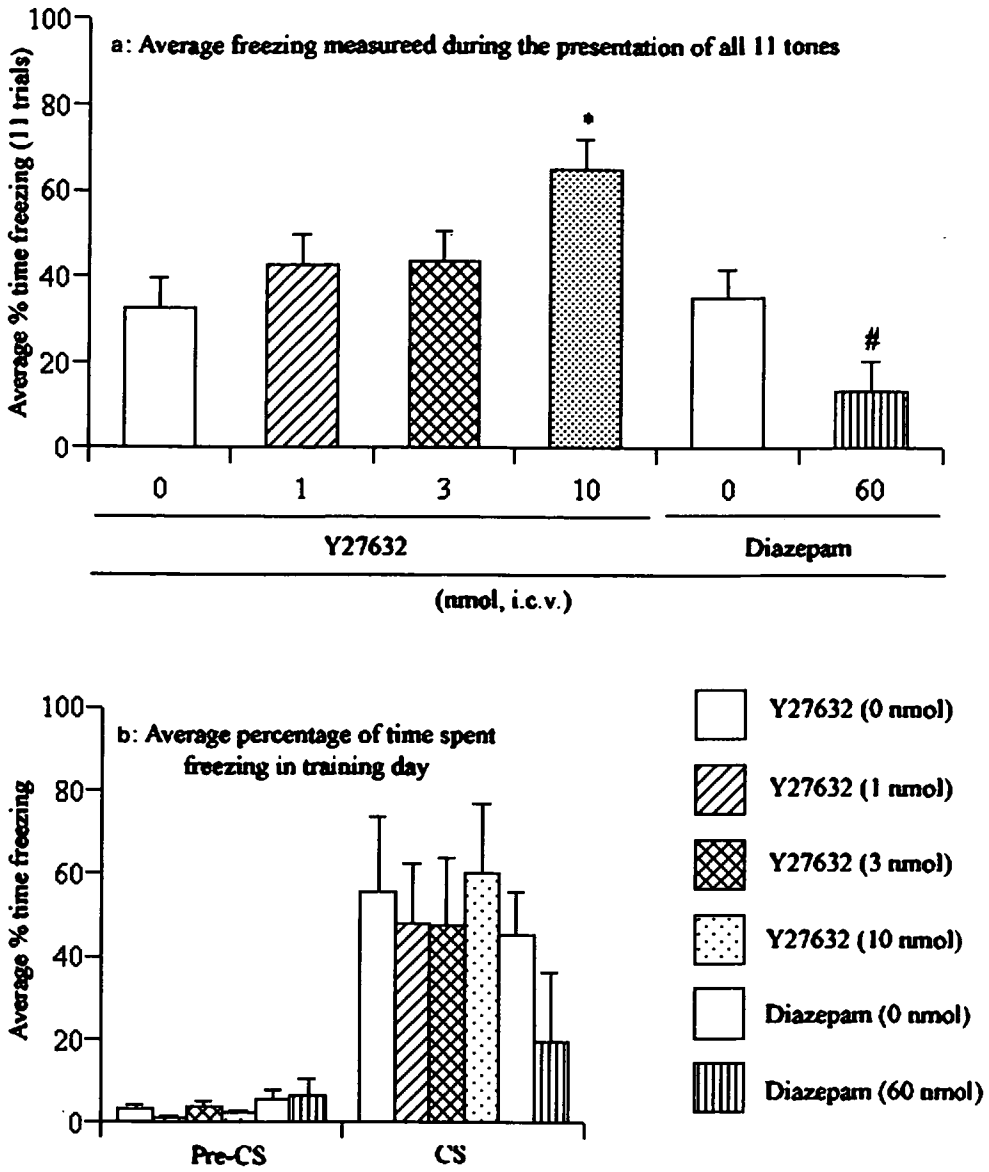



**Fig. 2** Effect of ROCK inhibition on fear-related behaviors in the fear conditioning test. *Panel* **a** summarizes the extent of average freezing that Y27632- and diazepam-treated mice displayed in response to the presentation of auditory fear-conditioned stimuli (11 tones). *Panel* **b** shows the percent freezing displayed by mice treated with either drugs or vehicle in response to an auditory fear-conditioned stimulus (CS). The pretone period (Pre-CS) represents the general motor activity of mice during the 360-s period before presentation of the first CS. CS represents freezing behavior of mice during the 240-s period of auditory fear conditioning. The data are expressed as means±S.E. The statistical significances of differences between Y27632 treatment groups were assessed with one-way analysis of variance (ANOVA), and post hoc individual group comparisons were made with Bonferroni/Dunn's test. The statistical significances of differences between diazepam treatment groups were assessed with a Student's *t* test. [Saline, $n$=10; 1 nmol, $n$=16; 3 nmol, $n$=16; 10 nmol, $n$=16]. *$P$<0.05 vs saline-treated mice (Y27632 treatment groups), #$P$<0.05 vs vehicle-treated mice (diazepam treatment groups)

revealed a higher response between the groups on the 10-nmol dose on the second, third, and fifth to seventh trials. In contrast, diazepam-treated mice (60 nmol, i.c.v.) froze significantly less often than did saline-treated mice during the presentation of all 11 tones (Fig. 2a). We also examined the effects of Y27632 posttraining treatment on freezing. Y27632 (10 nmol, i.c.v.) was injected into the mice immediately after the fear conditioning session. However, there was no significant effect in the freezing behavior of mice during the presentation of all 11 tones between Y27632- and vehicle-treated mice. (Table 1).

## Effects of ROCK inhibition on stress-related head-dipping behavior in the hole-board test

The effect of Y27632 on stress-related head-dipping behavior in mice is shown in Fig. 3. Figure 3a shows the effects of stress and drug on the head-dip counts of mice in the hole-board test. Two-way factorial ANOVA (stress × drug manipulation) revealed significant effects for stress × drug interaction [stress × drug interaction: $F(1,47)=4.182$, $P<0.05$]. Post hoc test analysis showed that stressing the mice acutely by restraining them for 30 min significantly decreased the number of head dips, and this effect in the head-dipping behavior produced by restraint stress was significantly reinforced by Y27632 treatment (10 nmol, i.c.v.). Figure 3b shows the effects of stress and drug on the head-dip latency of mice in the hole-board test. Two-way factorial ANOVA (stress × drug manipulation) revealed significant main effect for stress [stress: $F(1,47)=7.232$, $P<0.05$]. These analyses revealed that stressing the mice acutely by restraining them for 30 min significantly increased the latency of head-dipping behavior. In addition, post hoc test analysis showed that the latency to head dipping was significantly reinforced by

Table 1 The percent freezing displayed by mice posttreated with either Y27632 or vehicle in response to an auditory fear-conditioned stimulus

| Treatment | Dose (nmol, i.c.v.) | Time freezing (%) |
|---|---|---|
| Vehicle | 0 | 22.7±7.5 |
| Y27632 | 10 | 17.0±4.7 |

Y27632 (10 nmol, i.c.v.) was injected into the mice immediately after the fear conditioning session. Training consisted of five conditioning trials, which were five pairings of a 10-s tone conditioned stimulus (CS) (3 kHz, 85±1 dB) that coterminated with a footshock unconditioned stimulus (1.0 s, 1.5 mA). Mice were tested drug-free 24 h after they were conditioned. In test session, 11 presentations of the 10-s tone conditioned stimuli (intertrial interval, 20 s) were given. Fear-related behavior was evaluated by tracking the number of seconds mice froze during all 11 tones. Data represent the average freezing measured during the presentation of all 11 tones as the mean with S.E.M. of 8–10 mice

restraint stress. On the other hand, Y27632 (10 nmol) did not significantly affect the emotional behavior of nonstressed mice. Giving the mice diazepam (60 nmol, i.c.v.) before restraining them, however, reversed the restraint stress-induced decrease in head-dipping behavior. Y27632 and diazepam did not affect locomotor activity and rearing behavior in nonstressed or stressed mice (Fig. 3c,d).

Effects of ROCK inhibition on spontaneous alteration performance in the Y-maze test

Saline-treated mice had an average alteration score of 51.9±5.6%. In contrast, Y27632-treated mice (10 nmol, i.c.v.) had an average alteration score of 57.3±2.0%. Y27632 (10 nmol, i.c.v.) did not have significant effect on total arm entries (saline: 76.3±7.6 times; Y27632: 61.4±5.4 times).

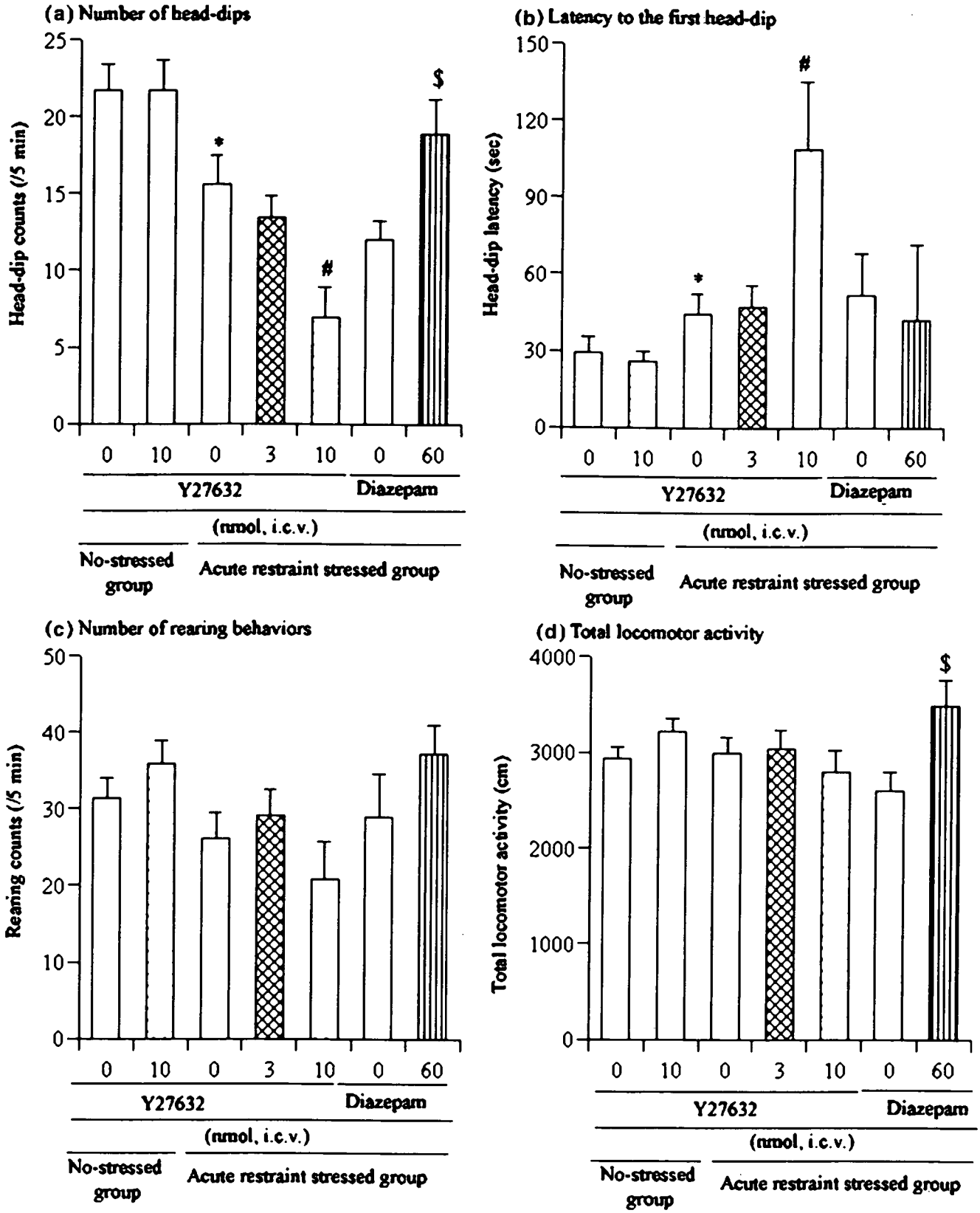
## Discussions

In the present study, the ROCK inhibitor Y27632 increased anxiety- and/or fear-related behaviors in mice performing an elevated plus-maze test, a fear conditioning test, and a hole-board test without changing the general locomotor activity of the treated mice. To our knowledge, this is the first demonstration that the Rho/ROCK pathway might be involved in the expression of anxiety- and/or fear-related behaviors. Elevated plus-maze test measures spontaneous, naturalistic behaviors within the innate repertoire of the animal because this test involves no conditioning (Handley and Mithani 1984; Pellow et al. 1985). Thus, one interpretation of the present finding in elevated plus-maze test is that mice become more fearful after administration of Y27632. On the other hand, contextual fear conditioning test measures the ability of mice to learn and remember an association between an aversive experience and environmental cues (Fanselow 1980). Thus, another interpretation of the finding in contextual fear conditioning test is that ROCK inhibition might enhance the acquisition of contextual fear conditioning.

Similar to exposure to anxiogenics, exposing mice to acute restraint stress has been shown to produce decreased head-dipping behavior and increased latency to head-dipping in the hole-board test (Rodriguez Echandia et al. 1987). In the present study, we confirmed these findings, observing similar behavioral suppression in mice evaluated with a hole-board test. Furthermore, we found that the suppression of head-dipping behavior was significantly reinforced by Y27632 at a dose that did not significantly affect the emotional behavior of nonstressed mice. Previously, it was reported that pretreatment with the 5-$HT_{1A}$ receptor agonists, but not benzodiazepine anxiolytics, 24 h before stress exposure suppressed the decrease in the head-dipping behaviors in mice in the hole-board test (Tsuji et al. 2000, 2001). On the other hand, it was also reported that 5-$HT_{1A}$ receptor agonists produced the decrease in the head-dipping behaviors in naive mice (Takeda et al. 1998). Thus, they suggested that 5-$HT_{1A}$ receptor agonists but not benzodiazepine anxiolytics may affect some adaptive mechanism(s) involved in the recognition of and/or ability to cope with stressful situation (Tsuji et al. 2000, 2001). Based on these results, we propose that Rho/ROCK pathway might involve some adaptive mechanism(s) involved in not only the expression of anxiety-related behaviors but also the recognition of and/or ability to cope with stressful situation.

During auditory fear conditioning and testing, we found no significant differences in the freezing behavior of saline- and Y27632-treated mice during the pretone period, indicating that both groups displayed similar levels of basal activity. Moreover, the levels of freezing behavior during auditory fear conditioning were not significantly different in mice given with Y27632 before training compared to those given with saline. These results indicate that Y27632-treated mice displayed neither motor impairment nor differences in pain threshold that could produce freezing-like behavior. Furthermore, on the elevated plus maze, the total number of entries into open and closed arms as well as locomotor activity was not significantly different in mice treated with the Y27632 dosages tested. In addition, Y27632 did not significantly affect spontaneous locomotor activity of nonstressed mice assessed on the hole-board test. Taken together, these observations demonstrate that anxiety- and/or fear-related behaviors in Y27632-treated mice did not result from general hyperactivity or sedation.

The present study showed that mice pretreated with diazepam froze significantly less than did mice treated with


(a) Number of head-dips
Head-dip counts (/5 min)
25
20
15
10
5
0
*
#
$
0 10 0 3 10 0 60
Y27632
Diazepam
(nmol, i.c.v.)
No-stressed group
Acute restraint stressed group
(b) Latency to the first head-dip
Head-dip latency (sec)
150
120
90
60
30
0
*
#
0 10 0 3 10 0 60
Y27632
Diazepam
(nmol, i.c.v.)
No-stressed group
Acute restraint stressed group
(c) Number of rearing behaviors
Rearing counts (/5 min)
50
40
30
20
10
0
0 10 0 3 10 0 60
Y27632
Diazepam
(nmol, i.c.v.)
No-stressed group
Acute restraint stressed group
(d) Total locomotor activity
Total locomotor activity (cm)
4000
3000
2000
1000
0
$
0 10 0 3 10 0 60
Y27632
Diazepam
(nmol, i.c.v.)
No-stressed group
Acute restraint stressed group

◀ **Fig. 3** Effects of ROCK inhibition on the changes in head-dipping behaviors produced by acute restraint stress on the hole board. Changes in the emotional state of mice were evaluated in terms of changes in exploratory activity, i.e., number of head dips (**a**), latency to the first head dip (**b**), number of rearing behaviors (**c**), and total locomotor activity (**d**). The data are expressed as means±S.E. The statistical significances of differences in hole-board test data between Y27632 treatment groups were assessed with two-way analysis of variance (ANOVA; stress × drug manipulation), and post hoc individual group comparisons were made with Bonferroni/Dunn's test. The statistical significances of differences between diazepam treatment group were assessed with a Student's *t* test. [Nonstressed group (saline, $n$=12; 10 nmol, $n$=11), stressed group (saline, $n$=12; 3 nmol, $n$=12, 10 nmol, $n$=12) or diazepam (vehicle, $n$=10; 60 nmol, $n$=10)]. *$P$<0.05 vs saline-treated nonstressed mice (Y27632 treatment groups), #$P$<0.05 vs saline-treated stressed mice (Y27632 treatment groups), and \$$P$<0.05 vs vehicle-treated mice (diazepam treatment groups)

vehicle in the fear conditioning test. It is said that the amnesia effect of diazepam also influences the freezing behavior in the fear conditioning test (Gravius et al. 2005). Indeed, the effects of diazepam on fear conditioning test might be concerned in amnesia as well as anxiolysis. Thus, to test whether ROCK inhibition influences memory and/or cognition, we examined the effects of Y27632 on the spontaneous alteration behavior of mice in the Y-maze test. We observed no significant difference in the spontaneous alteration behavior of mice treated with either saline or Y27632 (10 nmol, i.c.v.). Furthermore, Y27632 failed to produce any marked effects on total arm entries. These results suggest that the Y27632-induced freezing behavior we observed in the fear conditioning test was not influenced by spatial working memory and/or cognition. This supported our speculation that Y27632 enhanced fear-related behaviors after auditory-cued learning and contextual fear conditioning.

In the central nervous system, the Rho/ROCK pathway has been proposed to regulate dendritic and axonal morphology during development (Van Aelst and Cline 2004). Inhibition of the Rho/ROCK pathway may induce anxiety- and fear-related behaviors through a similar structural modification of neural processes involved in synaptic transmission and plasticity. It has been reported that the children with Williams syndrome, which is a neurodevelopmental disorder, showed higher rates of emotional difficulties such as concentration difficulties and excessive anxiety when compared with the normally developing children (Udewin and Yule 1991). Recently, it was suggested that the dysfunction of the LIM-kinase activity may contribute to emotional difficulties in Williams syndrome (Meyer-Lindenberg et al. 2005). In addition, it was demonstrated that LIM-kinase activity was regulated by ROCK (Maekawa et al. 1999). Thus, these results lead us to the possibility that the Rho/ROCK pathway, in particular downstream Rho effectors such as ROCK and LIM kinase, may underlie the expression of fear- and anxiety-related behaviors in rodents within the adolescent. However, further studies are necessary to clarify these effects of ROCK inhibitor.

Using a differential cloning strategy, we previously analyzed gene expression in rat frontal cortex after acute treatment with antidepressants (Yamada et al. 2000–2002; Yamada and Higuchi 2002; Nishioka et al. 2003; Takahashi et al. 2005). We found decreased expression of several Rho/ROCK pathway components in the frontal cortex of rats 24 h after a single administration of several different antidepressants (selective 5-HT reuptake inhibitors, 5-HT/norepinephrine reuptake inhibitors, and tricyclic antidepressants) when compared to the expression in the frontal cortex of control rats. Because of their efficacy and favorable side-effect profile, antidepressants are the treatment of choice for major depression and various anxiety disorders (Sheehan et al. 1993; Van der Kolk et al. 1994; Bezchlibnyk-Butler et al. 2000; Rickels and Rynn 2002). Paradoxical complications arise, however, because the acute clinical effects of antidepressants are sometimes opposite to their chronic therapeutic effects; that is, anxiety actually increases at the start of treatment and decreases only after several weeks of treatment (Gorman et al. 1987; Boyer and Feighner 1992; Goldstein and Goodnick 1998; Masand and Gupta 1999). Thus, these results suggest that acute antidepressant treatment may specifically affect the Rho/ROCK pathway in the frontal cortex. Our findings in the present study are in accord with those reported by Burghardt et al. (2004). It is interesting to note that they reported that antidepressant administration produced similar fear conditioning behaviors in rodents. Taken together with the clinical results discussed above, we propose that the Rho/ROCK pathway may similarly underlie the anxiety displayed by patients with depression or anxiety disorders. Furthermore, the anxiety-related behaviors commonly experienced by individuals at the start of antidepressant therapy may also involve the disruption of the Rho/ROCK pathway. However, further studies are necessary before these issues can be resolved unequivocally.

In conclusion, our findings demonstrate that ROCK is involved in the expression of anxiety- and/or fear-related behaviors. Furthermore, we propose that if the Rho/ROCK pathway plays an important role in mediating anxiety- and/or fear-related behaviors in humans, it may prove to be a novel system for anxiolytics to target.

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