because of medical illness; or (iv) medications, medical illness, physical disability, or cognitive dysfunction (Mini-Mental State Examination score <15) affecting their ability to perform the self-reporting test or to provide consent.

Data were gathered from a subset of the subjects of a previous study, which was a research project on depression and lesion location, or depression/apathy and functional outcome, or sitting balance among stroke rehabilitation patients.[11–13] Patients were not selected on the basis of the results of the previous study.

Treatment

The unit at Nishi-Hiroshima Rehabilitation Hospital provides intensive multidisciplinary goal-oriented inpatient rehabilitation. Every 1 or 2 weeks, the staff, including medical doctors, nurses, care workers (CW), physical therapists (PT), occupational therapists (OT), speech therapists (ST), medical social counselors (MSW) and clinical psychologist (CP), assemble in the conference room and make arrangements regarding the physical, psychological, or social problems of each inpatient, and review the patient rehabilitation programs.

Computed tomography

CT scanning was carried out for all patients on admission; a follow-up CT scan was performed every 1–3 months after admission to measure the infarction/hemorrhage site and volume (cubic centimeters) according to the formula 0.5 A B C, where A and B represent the largest perpendicular diameters through the hypodense area on the CT scan, and C is the thickness of the infarction area.[11–13]

Functional measures

The Functional Independence Measurement (FIM; version 3.0) is an observer-rated multi-item summed rating scale used to evaluate disability in terms of dependency, and is widely used as a measure of disability in stroke patients.[12–15] The maximum total FIM score is 126; the lower the score, the greater the disability. All patients were examined for disability using the FIM (Japanese version) within 1 week after admission and at 1–2-week intervals during hospitalization.

The improvement in FIM score per week during hospitalization was calculated as follows: [(FIM score on discharge) (FIM score on admission)]/[period of hospitalization (weeks)].

Motor impairment in hemiplegic stroke patients was measured by the stage on the Brunnstrom Recovery Scale (BRS), in which movement patterns are evaluated and motor function is rated according to stages of motor recovery.[12,13,16] The BRS scale defines recovery only in broad categories; these categories correlate with progressive functional recovery.

Psychological assessments: process of acceptance of disability and insistence on recovery

Inpatient psychological status (acceptance stage and insistence on recovery) was assessed by observation of the behavior of patients under the guidance of clinical psychologists. Information relating to patients' psychological complaints varies among staff members, because the patient usually does not convey his or her real feelings equally to all staff. The acceptance stage and insistence on recovery were therefore estimated on the basis of statements by every doctor, nurse, CW, PT, OT, ST, MSW and CP.

The stage of acceptance of disability in each inpatient was estimated using Fink's theory of the acceptance process (first stage, shock; second stage, defensive retreat; third stage, acknowledgement; fourth stage, acceptance and change) as described previously.[7] In scoring the acceptance stage, a value of 1–4 was assigned to an observation as follows: shock, 1; defensive retreat, 2; acknowledgement, 3; acceptance and change, 4.

'Insistence on recovery' was defined as the patient's direct verbal report or, as inferred from their behavior, that they thought that they would soon get well.[8] The assumption of a normal body is implicit in any discussion of future plans. The person is preoccupied with their physical condition and is apt to overestimate the meaning of any small improvement. They say, 'I know it's taken a long time, but I still haven't given up hope. 'Insistence on recovery' was estimated by observation of patient behavior in quantitative terms: the 'insistence on recovery' score was constructed on a scale on which complaints that are noted a little of the time, some of the time, much of the time, or most of the time, were scored 1, 2, 3, and 4, respectively.

Self-rating Depression Scale

We used the Japanese version of the SDS to examine the subjective severity of depression.[11–13,17] Patients completed the SDS within 1 month after admission. We classified the patients into two groups according to their score: a non-depressed group (SDS score <45 points) and a depressed group (SDS score ≥45 points). The cut-off point was determined on the basis of a previous report on Japanese stroke patients.[17]

Apathy Scale

To quantify the apathetic state, we used a Japanese version of the AS.[11–13,18–20] Patients completed the AS within 1 month after admission. The AS consists of 14 questions concerning spontaneity, initiation, emotionality, activity level, and interest in hobbies. This scale was self-assessed. The answers to each question were scored as 0–3 and the total score was used for the analysis. We classified the patients into two groups according to their score: a non-apathetic group (AS < 16 points) and an apathetic group (AS ≥ 16 points).

Statistical analysis

Statistical analyses were based on the assumption that the data were not normally distributed, analysis being performed with non-parametric tests to examine the correlation between the middle-aged and elderly groups; Fisher's exact tests were used to compare categorical variables and the Mann-Whitney U test was used to compare continuous variables.

Differences in the time course of acceptance of disability or FIM score were assessed using Kruskal–Wallis one-way analysis of variance (ANOVA) at admission, 3 months, and 5 months. Post-hoc testing was performed using the Scheffé test.

Multiple regression was used to estimate the independent effects of predictor variables (highest attained acceptance stage, 'insistence on recovery' score, age, sex, presence of a history of stroke, BRS, FIM score on admission, period of hospitalization) on improvement in FIM (FIM gain/week).

Different degrees of the acceptance stage or insistence on recovery stage were compared with the SDS or AS score using one-way ANOVA followed by a post-hoc Fisher protected least significant difference test.

Values were considered to be significant at $P < 0.05$. Stat View 5.0 (SAS Institute, Cary, NC, USA) was used for all analyses.

RESULTS

Baseline patient data

Table 1 lists the baseline data for all patients in the two age groups (middle-aged or elderly). There were

Table 1. Baseline data for stroke inpatients

	Total (n = 231)	Middle-aged (n = 95)	Elderly (n = 136)	P
Age (years)	66.3 ± 10.2	56.2 ± 6.1	73.4 ± 5.4	<0.0001
Gender: male/female	162/69	77/18	85/51	0.0033
Type of stroke: hemorrhage/infarction	95/136	47/48	48/88	0.0413
Presence of history of stroke, n (%)	38 (16.5)	11 (11.6)	27 (19.9)	0.1069
Period of hospitalization, days	152.5 ± 51.2	146.8 ± 60.4	160.4 ± 49.7	0.0806
Side of stroke: right/left/bilateral	101/109/21	40/47/8	61/62/13	0.8373
Size of CT finding (cm^3)	37.9 ± 55.6	38.2 ± 59.0	37.6 ± 53.4	0.9614
FIM score on admission	64.1 ± 25.0	69.7 ± 25.1	61.1 ± 24.5	0.0003
FIM score on discharge	84.5 ± 25.5	90.9 ± 22.5	81.1 ± 26.4	0.0002
FIM gain/week	0.86 ± 0.56	0.87 ± 0.56	0.84 ± 0.56	0.4963
Stage of acceptance at admission	1.9 ± 1.0	1.9 ± 1.0	1.8 ± 1.1	0.3548
Stage of acceptance at discharge	3.0 ± 1.1	3.1 ± 1.1	3.0 ± 1.2	0.4822
Insistence on recovery score at admission	2.0 ± 0.9	2.2 ± 1.0	1.9 ± 0.9	0.093
Insistence on recovery score at discharge	2.1 ± 0.9	2.3 ± 0.9	2.0 ± 0.8	0.0019

Fisher's exact test was used to compare categorical variables; the Mann–Whitney U-test was used to compare continuous variables, and to test correlation between the middle-aged and elderly groups.
FIM, Functional Independence Measurement.

Table 2. Time course of FIM, stage of acceptance and insistence on recovery score during 5 months after admission

			Middle-aged group				Elderly group			
			Admission (n = 95)	3 months (n = 76)	5 months (n = 52)	Kruskal-Wallis	Admission (n = 136)	3 months (n = 118)	5 months (n = 95)	Kruskal-Wallis
FIM	Score		78.2 ± 25.9	86.4 ± 23.0	89.5 ± 20.5	$P < 0.0001$	65.0 ± 26.4	76.0 ± 27.1	78.2 ± 24.6	$P < 0.0001$
	Scheffé	Admission vs 3 months	$P = 0.0007$				$P = 0.0002$			
		Admission vs 5 months	$P < 0.0001$				$P < 0.0001$			
		3 months vs 5 months	$P = 0.1667$				$P = 0.4095$			
Acceptance of disability	Stage	1 Shock, n(%)	40 (42.1)	12 (15.8)	5 (9.6)	$P < 0.0001$	71 (52.2)	31 (26.3)	18 (18.9)	$P < 0.0001$
		2 Defensive retreat, n(%)	34 (35.8)	15 (19.7)	6 (11.5)		34 (25.0)	19 (16.1)	11 (11.6)	
		3 Acknowledgment, n(%)	9 (9.5)	16 (21.1)	9 (17.3)		10 (7.4)	22 (18.6)	14 (14.7)	
		4 Acceptance and change, n(%)	10 (10.5)	33 (43.4)	32 (61.5)		19 (14.0)	46 (39.0)	52 (54.7)	
		ND, n (%)	2 (2.1)	0	0		2 (1.5)	0	0	
	Scheffé	Admission vs 3 months	$P < 0.0001$				$P < 0.0001$			
		Admission vs 5 months	$P < 0.0001$				$P < 0.0001$			
		3 months vs 5 months	$P = 0.0690$				$P = 0.0905$			
Insistence on recovery	Score	1, n (%)	24 (25.3)	17 (22.4)	11 (21.2)	$P = 0.5259$	44 (32.4)	42 (35.6)	27 (28.4)	$P = 0.7386$
		2, n (%)	16 (16.8)	26 (34.2)	20 (38.5)		30 (22.1)	40 (33.9)	38 (40.0)	
		3, n (%)	27 (28.4)	26 (34.2)	15 (28.8)		34 (25.0)	28 (23.7)	23 (24.2)	
		4, n (%)	5 (5.3)	4 (5.3)	4 (7.7)		1 (0.7)	2 (1.7)	3 (3.2)	
		ND, n (%)	23 (24.2)	3 (3.9)	2 (3.8)		27 (19.9)	6 (5.1)	4 (4.2)	
	Scheffé	Admission vs 3 months	$P = 0.6334$				$P = 0.9393$			
		Admission vs 5 months	$P = 0.5732$				$P = 0.7451$			
		3 months vs 5 months	$P = 0.9939$				$P = 0.9098$			

Differences in time course (e.g. at admission, 3 months and 5 months) of acceptance of disability, FIM score, or insistence on recovery were assessed using Kruskal–Wallis one-way ANOVA. Post-hoc tests were done using the Scheffé test.
FIM, functional independence measurement; ND, not determined.

no differences in the presence of stroke history, laterality of the stroke, size of CT findings, FIM gain/week, acceptance stage, or insistence on recovery score at admission between the two age groups at baseline. The two age groups were not matched for sex, type of stroke, FIM score, or insistence on recovery score at discharge. The FIM score, insistence on recovery score at discharge, male gender and rate of hemorrhage were much higher in the middle-aged group than in the elderly group.

Time course of the acceptance stage, insistence on recovery score and FIM score

Changes in the FIM score, acceptance stage, and insistence on recovery score over time are given in Table 2. In both age groups the acceptance stage progressed and the FIM score increased significantly each month ($P < 0.0001$, Kruskal–Wallis test). Post-hoc testing (Scheffé) indicated a difference between the acceptance stage at admission and at 3 or 5 months. But we found no significant differences between acceptance stage or FIM score at 3 months or at 5 months (Scheffé). We found no differences in the insistence on recovery score on admission, at 3 months or at 5 months (Kruskal–Wallis test and Scheffé test). Therefore, progression of acceptance stage and functional improvement were evident, especially during the first 3 months after admission, but the insistence on recovery score did not change during hospitalization in either age group.

Effects of acceptance stage or insistence on recovery score on improvement in FIM after a stroke

To identify predictors of improvement in FIM after a stroke, we performed multiple regression using sex, age, presence of history of stroke, period of

Table 3. Multiple regression for FIM gain/week

	FIM gain/week			
	Middle-aged		Elderly	
	SC	*P*	SC	*P*
Acceptance stage	0.078	0.5213	0.091	0.381
Insistence on recovery score	0.07	0.5551	0.218	0.0348
BRS upper limb	0.083	0.7871	0.314	0.1487
BRS finger	0.236	0.3957	0.215	0.3025
BRS lower limb	0.193	0.3284	0.258	0.0833
CT size	0.034	0.7877	0.122	0.2084
Presence of a history of stroke	0.049	0.6734	0.084	0.3743
Period of hospitalization	0.113	0.4671	0.222	0.0309
Age	0.127	0.287	0.151	0.098
Sex	0.045	0.692	0.055	0.545
FIM score on admission	0.288	0.1123	0.341	0.0073

BRS, Brunnstrom Recovery Scale; CT, computed tomography; FIM, Functional Independence Measurement; SC, standardized coefficient.

hospitalization, FIM at admission, BRS (upper limb, finger, lower limb), acceptance stage and insistence on recovery score as independent variables, with improvement in FIM as the dependent variable (Table 3). In the middle-aged group, no predictors were found. In the elderly group, however, the FIM score on admission, the period of hospitalization, and the insistence on recovery score were correlated significantly with FIM gain/week. It was noteworthy that the insistence on recovery score (but not the acceptance stage) correlated positively with improvement in FIM in the elderly group.

Effects of acceptance stage or insistence on recovery score on depression or apathy after a stroke

To examine the effects of the acceptance stage on depression or apathy, we used ANOVA and post-hoc test (Fig. 1). The SDS score (Fig. 1a) and AS score (Fig. 1c) changed significantly from 'shock (first) stage' to 'acceptance and change (fourth) stage'. Post-hoc testing indicated a difference between first and fourth, second and third, and third and fourth stages for the SDS score and between first and fourth, and third and fourth stages for the AS score. Both SDS and AS scores were highest in the third stage but significantly decreased in the fourth stage.

The SDS score (Fig. 1b) and AS score (Fig. 1d) changed significantly as insistence on recovery score changed from 1 to 4. On post-hoc testing there were significant differences between insistence on recovery scores 1 and 3, and insistence on recovery score 3 and 4 for the SDS score, and significant differences between insistence on recovery scores 1 and 2, and insistence on recovery scores 1 and 3 on the AS score. It is noteworthy that both the SDS and AS scores decreased as insistence on recovery score increased from 1 to 3, and then increased for insistence on recovery scores 3–4.

DISCUSSION

The present results demonstrate that many stroke patients improved in functional disability, proportional to progress in stage of acceptance of disability in the rehabilitation hospital. It is surprising that the presence of insistence on recovery enhanced functional improvement. To our knowledge this is the first stroke study to address the influence of insistence on recovery on functional improvement after a stroke.

Stage of acceptance of disability correlated with FIM improvement

The stage theory of acceptance of disability states that people undergoing a life crisis follow a predictable, orderly path of emotional response. In the present study we examined the effect of acceptance on func-

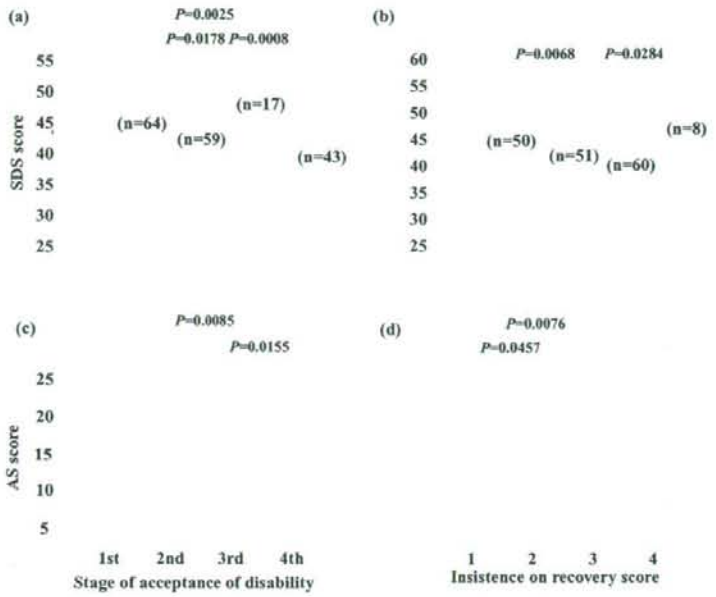

Figure 1. Differences in (a,b) Zung Self-rating Depression Scale (SDS) and (c,d) Apathy Scale (AS) score according to (a,c) acceptance stage (first, second, third, and fourth) and (b,d) insistence on recovery score (1–4). The data points are given as mean and 95% confidence interval. The Fisher protected least significant difference test also indicates that these parameters can distinguish between some of these psychological subgroups. (a) $P = 0.0018$; (b) $P = 0.0195$; (c) $P = 0.0284$; (d) $P = 0.0370$ (all ANOVA).

tional improvement in Japanese stroke patients. The present data demonstrated that acceptance stage progress and FIM scores increased significantly each month, particularly in the first 3 months after hospital admission (Table 2). At the onset of physical disability after a stroke, the individual cannot tolerate the overwhelming chaos accompanying the shock.[7,8] In this shock phase, the person feels emotionally numb and experiences a sense of depersonalization. Physical recovery from the acute phase is interpreted as a sign that everything is returning to its former state.[7,8] At that time, the acceptance stage progresses from the shock phase to defensive retreat. When the disabled patient gradually begins to experience a physical plateau, the acknowledgement phase occurs.[7,8,21,22] The patient no longer finds it possible to escape reality and experiences the loss of their valued self-image. The feeling-state, which accompanies these changes, is one of deep depression as in mourning. Therefore, patients in this acknowledgement stage suffered higher levels of depression and apathy than those in the other acceptance stages (Fig. 1). The patient who has accepted their permanent physical impairment considers the disability to be merely one of their many characteristics.[7,8,21,22] Therefore, it was suggested that many stroke patients functionally improved in parallel with progression of the acceptance stage.

Appropriate level of insistence on recovery reduced depression and apathy, resulting in an improvement in the FIM

According to the stage theory of acceptance, insistence on recovery is a sign of denial, and an indicator of poor prognosis in rehabilitation.[5–8] But the present data contradicted this; the appropriate level of insistence on recovery reduced depression and apathy, resulting in an improvement in the FIM. The question arises as to the nature of insistence on recovery in the present study.

Changes in physical functioning or appearance must be incorporated into a revised self-image, which can necessitate a change in personal values and lifestyle.[23–25] The individual must then prepare for an uncertain future with the threat of permanent physical disabilities, which results in a deep depression, similar to mourning. To cope with this identity crisis, individuals must maintain hope that restoration of function is possible.[24] Even when the prognosis is certain, the future is still uncertain; patients think about their physical disability and hope for improvement every day (the so-called 'insistence on recovery' in the present study). The disabled stroke patients experience these positive (restoration of function, maintain hope) and negative (disability would continue permanently) feelings toward their disability in turn. The coexistence of both positive and negative feelings is commonly understood as ambivalence,[26] and representation of insistence on recovery was thought to be a sign of post-stroke ambivalent state during the mourning process. In the traditional view, ambivalence has been seen as particularly important to the development of complicated grief, but Piper *et al* reported the opposite result: the more ambivalent the behavior of the patient, the less severe was the grief.[26] Defining of their disability is a painful process for the stroke patient. But insistence on recovery (ambivalence) may minimize the seriousness of the crisis (permanent physical disability, identity crisis) and reduce the pain during the process of defining the disability. Therefore, many stroke patients can confront this painful mourning process (defining their disability) gradually, in order to keep the depressive or apathetic symptoms to a minimum, easing the pain with the help of a more optimistic idea ('insistence on recovery' or ambivalent feeling). Thus insistence on recovery may be considered as part of the fighting spirit in which patients seek to conquer disease (such as cancer) based on hope, and indicates a good prognosis.[27–29] Judging from these observations it is possible that insistence on recovery in the present study may be a favorable prognostic factor for disabled stroke patients.

Severe level of 'insistence on recovery' associated with both depression and apathy

Disabled stroke patients with a severe level of insistence on recovery form only a minority of stroke patients, but they suffered severe depression and apathy according to the present data. Insistence on recovery is thought to be a sign of denial. Denial is generally found in cognitive psychological research of psychopathology, and sometimes denial is found in non-psychological patients. Mildly depressed individuals are more balanced in self-perceptions and evince more accurate predictions of control and future outcomes.[6] More severe depression often yields negative appraisal tendencies.[6] The patients with severe insistence on recovery are thought to be in a severe denial state and therefore simply wait for recovery, and often state that they do not understand the purpose of rehabilitation exercises, resulting in poor participation in rehabilitation therapy.[30–32]

In a rehabilitation unit many staff feel that these patients are troublesome. Although patients with severe levels of insistence on recovery are only in a minority, their characteristics are conspicuous, and all staff tend to think empirically that the existence of insistence on recovery prevents an improvement in functional disability, irrespective of the degree of severity. This staff tendency was thought to be a countertransference reaction, which generates more negative interactions with patients, leading to worse outcome.[33] In practice the majority of patients have an appropriately low level of insistence on recovery, which helps disabled patients to confront the painful acceptance process (reduce depression and apathy). But encouraging the patients to give up insistence on recovery regardless of the possible low level of severity, might also reduce the protection that stroke patients have from depression or apathy, resulting in preventing stroke patients from improving their functioning.

Disparity in functional and psychological states between middle-aged and elderly patients

In the present study, insistence on recovery enhanced functional improvement after a stroke: this trend was statistically significant in the elderly group, but not in the middle-aged group. A question arises regarding the difference between the middle-aged and elderly groups. Once an individual reaches old age, the body starts to lose its autonomy.[9,10] As independence and control are challenged, self-esteem and confidence weaken. Most elderly people seem to find themselves, almost involuntarily, thinking about dying and about feeling ill, depressed, and somehow let down.[10,34,35] To some extent, these thoughts reflect a desperation that confronts all older people. But most people

struggle to counterbalance these associations with thoughts of more optimistic, life-affirming involvement.[34,35] These observations suggest that elderly stroke patients hope for recovery from their physical disability, and try to counterbalance desperation with thoughts of more optimistic, life-affirming involvement. Therefore insistence on recovery may encourage elderly stroke patients to participate in a rehabilitation program and gain functional improvement during hospitalization.

Study limitations

The present findings do not suggest that insistence on recovery causes depression and apathy; rather they indicate that insistence on recovery is frequently associated with depression and apathy, and likely interacts with the recovery process. The present findings should be seen in the light of certain methodological limitations. First, the sample size was small and the number of patients with a severe level of insistence on recovery was limited. Therefore the results require replication with a larger sample. Second, no structured personality scale was used; hence personality data might have been influenced by recall bias. Third, there is a possibility that social factors, such as employment and economic problems, might be more strongly influenced than psychological problems in the present study, and thus insistence on recovery might not influence ADL improvement in the middle-aged group. Fourth, the present results refer to national characteristics of Japanese people, and thus are not typical of other countries. Fifth, the psychological measurements in the present report were carried out only within 6–9 months after the onset of stroke, but the process of acceptance or mourning against disability is thought to continue for many years after the onset of stroke. Therefore, further longitudinal study is required to clarify the long-term effect of acceptance or insistence on recovery on the improvement of ADL and social function after the onset of stroke.

CONCLUSIONS

Progression of acceptance stages kept pace with improvement in functional disability after a stroke during rehabilitation. A mild level of insistence on recovery is a kind of fighting spirit, a rational belief, which minimizes the seriousness of the integrity of self-image, and accelerates functional improvement. A severe (not mild) level of insistence on recovery, however, is an irrational belief, and leads patients to develop a more severe depressive state. Insistence on recovery was previously believed to be a negative indicator for functional improvement of disabled stroke patients, but the present data contradict this, especially among elderly patients. Thus, the clinician should be aware of the severity of the patient's insistence on recovery in order to facilitate improvement of ADL especially among elderly stroke patients. When caring for patients, especially elderly patients, we should inform them of their prognosis in such a way such that they do not give up hope.

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REFERENCES

1 Roth EJ, Harvey RL. Rehabilitation of stroke syndromes. **In**: Braddom RL (ed.). *Physical Medicine and Rehabilitation*, 2nd edn. WB Saunders, Philadelphia, PA, 2000; 1117–1153.

2 Anderson TP. Rehabilitation of patients with completed stroke. **In**: Kottke FJ, Lehmann JF (eds). *Krusen's Handbook of Physical Medicine and Rehabilitation*, 4th edn. WB Saunders, Philadelphia, PA, 1990; 656–677.

3 Kelly-Hayes M, Paige C. Assessment and psychologic factors in stroke rehabilitation. *Neurology* 1995; **45**: S29–S32.

4 Han L, Law-Gibson D, Reding M. Neurological impairments influence function-related group outcomes after stroke. *Stroke* 2002; **33**: 1920–1924.

5 Rohe DE. Psychological aspects of rehabilitation. **In**: DeLisa JA, Gans BM (eds). *Rehabilitation Medicine: Principles and Practice*, 3rd edn. Lippincott Williams & Wilkins, Philadelphia, PA, 1998; 189–212.

6 Trexler LE, Fordyce DJ. Psychological perspectives on rehabilitation: Contemporary assessment and intervention strategies. **In**: Braddom RL (ed.). *Physical Medicine and Rehabilitation*, 2nd edn. WB Saunders, Philadelphia, PA, 2000; 75–92.

7 Fink SL. Crisis and motivation: A theoretical model. *Arch. Phys. Med. Rehabil.* 1967; **48**: 592–597.

8 Cohn N. Understanding the process of adjustment to disability. *J. Rehabil.* 1961; **27**: 16–18.

9 Clark GS, Siebens HC. Geriatric rehabilitation. **In**: DeLisa JA, Gans BM (eds). *Rehabilitation Medicine: Principles and Practice*, 3rd edn. Lippincott Williams & Wilkins, Philadelphia, PA, 1998; 963–995.

[10] Ganguli M, Dodge HH, Mulsant BH. Rates and predictors of mortality in an aging, rural, community-based cohort: The role of depression. *Arch. Gen. Psychiatry* 2002; **59**: 1046–1052.
[11] Hama S, Yamashita H, Shigenobu M *et al.* Post-stroke affective or apathetic depression and lesion location: Left frontal lobe and bilateral basal ganglia. *Eur. Arch. Psychiatry Clin. Neurosci.* 2007; **257**: 149–152.
[12] Hama S, Yamashita H, Shigenobu M *et al.* Depression or apathy and functional recovery after stroke. *Int. J. Geriat. Psychiatry* 2007; **22**: 1046–1051.
[13] Hama S, Yamashita H, Shigenobu M *et al.* Sitting balance as an early predictor of functional improvement in association with depressive symptoms in stroke patients. *Psychiatry Clin. Neurosci.* 2007; **61**: 543–551.
[14] Data Management Service of the Uniform Data System for Medical Rehabilitation and the Center for Functional Assessment Research. *Guide for Use of the Uniform Data Set for Medical Rehabilitation Including the Functional Independence Measure (FIM)*, version 3.0. State University of New York, Buffalo, NY, 1990.
[15] Tsuji T, Liu M, Sonoda S, Domen K, Chino N. The stroke impairment assessment set: Its internal consistency and predictive validity. *Arch. Phys. Med. Rehabil.* 2000; **81**: 863–868.
[16] Brandstater ME. Stroke rehabilitation. **In**: DeLisa JA, Gans BM (eds). *Rehabilitation Medicine: Principles and Practice*, 3rd edn. Lippincott-Raven, Philadelphia, PA, 1998; 1165–1189.
[17] Yamaguchi S, Kobayashi S, Koide H, Tsunematsu T. Longitudinal study of regional cerebral blood flow changes in depression after stroke. *Stroke* 1992; **23**: 1716–1722.
[18] Okada K, Kobayashi S, Yamagata S, Takahashi K, Yamaguchi S. Poststroke apathy and regional cerebral blood flow. *Stroke* 1997; **28**: 2437–2441.
[19] Starkstein SE, Fedoroff JP, Price TR, Leiguarda R, Robinson RG. Apathy following cerebrovascular lesions. *Stroke* 1993; **24**: 1625–1630.
[20] Yamagata S, Yamaguchi S, Kobayashi S. Impaired novelty processing in apathy after subcortical stroke. *Stroke* 2004; **35**: 1935–1940.
[21] Wright BA. *Physical Disability: A Psychological Approach*, 2nd edn. Harper & Row, New York, NY, 1960; 106–137.
[22] Shontz FC. Psychological adjustment to physical disability: Trends in theories. *Arch. Phys. Med. Rehabil.* 1978; **59**: 251–254.
[23] Horn S. Towards a therapeutic alliance model of rehabilitation. **In**: Wilson BA, McLellan DL (eds). *Rehabilitation Studies Handbook*. Cambridge University Press, Cambridge, 1997; 75–93.
[24] Moos RH, Tsu VD. *The Crisis of Physical Illness: An Overview*. Plenum Medical Book, New York, 1977; 3–21.
[25] Ellis-Hill CS, Horn S. Change in identity and self-concept: A new theoretical approach to recovery following a stroke. *Clin. Rehabil.* 2000; **14**: 279–287.
[26] Piper WE, Ogrodniczuk JS, Joyce AS, Mccallum M, Weideman R, Azim H. Ambivalence and other relationship predictors of grief in psychiatric outpatients. *J. Nerv. Ment. Dis.* 2001; **189**: 781–787.
[27] Penson RT, Talsania SH, Chabner BA, Lynch TJ Jr. Help me help you: Support groups in cancer therapy. *Oncologist* 2004; **9**: 217–225.
[28] Greer S, Morris T, Pettingale KW. Psychological response to breast cancer: Effect on outcome. *Lancet* 1979; **2**: 785–787.
[29] Pettingale KW, Morris T, Greer S, Haybittle JL. Mental attitudes to cancer: An additional prognostic factor. *Lancet* 1985; **1**: 750.
[30] Lenze EJ, Munin MC, Quear T *et al.* The Pittsburgh Rehabilitation Participation Scale: Reliability and validity of a clinician-rated measure of participation in acute rehabilitation. *Arch. Phys. Med. Rehabil.* 2004; **85**: 380–384.
[31] Maclean N, Pound P, Wolfe C, Rudd A. Qualitative analysis of stroke patients' motivation for rehabilitation. *BMJ* 2000; **321**: 1051–1054.
[32] Maclean N, Pound P, Wolfe C, Rudd A. The concept of patient motivation: A qualitative analysis of stroke professionals' attitudes. *Stroke* 2002; **33**: 444–448.
[33] Holmqvist R, Armelius K. Countertransference feelings and the psychiatric staff's self-image. *J. Clin. Psychol.* 2000; **56**: 475–490.
[34] Erikson EH, Erikson JM, Kivnick HQ. *Vital Involvement in Old Age*. WW Norton, New York, 1989.
[35] Erikson EH, Erikson JM. *The Life Cycle Completed*. WW Norton, New York, 1998.

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Research report

Neonatal tactile stimulation reverses the effect of neonatal isolation on open-field and anxiety-like behavior, and pain sensitivity in male and female adult Sprague–Dawley rats

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Abstract

It is well known that early life events induce long-lasting psychophysiological and psychobiological influences in later life. In rodent studies, environmental enrichment after weaning prevents the adulthood behavioral and emotional disturbances in response to early adversities. We compared the behavioral effect of neonatal isolation (NI) with the effect of NI accompanied by tactile stimulation (NTS) to determine whether NTS could reverse or prevent the effects of NI on the adulthood behavioral and emotional responses to environmental stimuli. In addition, we also examined the sex difference of the NTS effect. Measurements of body weights, an open-field locomotor test, an elevated plus maze test, a hot-plate test, and a contextual fear-conditioning test were performed on postnatal day 60. As compared with rats subjected to NI, rats subjected to NTS showed significantly higher activity and exploration in the open-field locomotor test, lower anxiety-like behavior in the elevated plus maze test, and significantly prolonged latencies in the hot-plate test, and this effect was equal among males and females. In the contextual fear-conditioning test, whereas NTS significantly reduced the enhanced freezing time due to NI in females, no significant difference in the freezing time between NI and NTS was found in males. These findings indicate that adequate tactile stimulation in early life plays an important role in the prevention of disturbances in the behavioral and emotional responses to environmental stimuli in adulthood induced by early adverse experiences.

Keywords: Neonatal isolation (NI); Neonatal tactile stimulation (NTS); Rat; Open-field locomotor test; Elevated plus maze test; Hot-plate test; Contextual fear-conditioning test

1. Introduction

It is well known that early experiences induce long-lasting psychophysiological and psychobiological changes in later life. Numerous studies have demonstrated that early adverse experiences such as maternal separation (MS) or neonatal isolation (NI), which interrupt dam–pup interactions, affect the development of the central nervous system and subsequently lead to enhanced susceptibility to stress in adulthood, both behaviorally and endocrinologically [8,10,12,14,17,37]. NI and MS differ with respect to isolation of individual offspring. With most MS procedures, only the dams are removed to separate cages, while the pups remain in their home cage. With NI procedures, pups are isolated and placed individually into containers separately from their dams and littermates. In contrast, the protective or therapeutic effects of early intervention on the development of stress vulnerability during the interruption of the dam–pup relationship has not been as thoroughly examined. For example, brief handling of neonatal rats during maternal separation was reported to induce resistance of the hypothalamo-pituitary-adrenal (HPA) axis to stress in adult rats [17]. Several studies have demonstrated that adult rats subjected to neonatal handling exhibited less anxiety-like behaviors in the elevated plus maze as compared with nonhandled rats [19,27].

Another type of neonatal handling, neonatal tactile stimulation (NTS), also has a distinct effect on the development of stress reactivity. Rats subjected to NTS show increased curiosity and problem-solving ability, and exhibit less emotionality in stressful situations [16]. NTS prevents the rise of serum corticosterone

Abbreviations: NI, neonatal isolation; NTS, neonatal tactile stimulation

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levels typically associated with maternal deprivation stress and causes elevated corticosterone levels to return to normal [25]. NTS also protects against maternal deprivation-induced shortening of hot-plate latencies [35]. Furthermore, it has been reported that NTS alleviates the reduction of hippocampal volume in rats subjected to neonatal hypoxia–ischemia [29]. These findings indicate that subjecting animals to NTS can facilitate their ability to cope with stressful environmental conditions, decrease the enhanced HPA axis in response to stress in adulthood, and protect against brain damage induced by neonatal manipulation.

Postnatal handling, which involves only a brief period (15 min) of mother–pup separation, dampens HPA responses to stress [15,20,21]. In contrast, postnatal MS (3 h/day; PN days 2–14) or (6 h/day; PN days 2–10) enhances HPA responses to stressors [18,28]. In addition, 1 h-neonatal isolation on postnatal days 2–9 also enhances HPA responses to stressors [3]. It has also been shown that early adverse experiences have sex-specific effects on the development of HPA-axis reactivity [4,36]. Similarly, gender differences exist with respect to the effects of neonatal isolation and neonatal handling on the development of anxiety-like behavior in the elevated plus maze [8,19] and the conditioned fear test [1,11]. Although there was no significant sex differences in anxiety-like behavior between rats subjected to neonatal handling and neonatal handling with tactile stimulation [32], it is unclear whether sex differences exist with respect to the ability of NTS to prevent or reverse the enhancement of susceptibility to environmental stimuli in response to early adversities.

In this context, the present study was undertaken to assess whether NTS can prevent or reverse the enhanced susceptibility to environmental stimuli due to NI in adult rats. We compared the effect of NI with the effect of NTS during NI on body weights, locomotor activities in the open-field test, anxiety-like behavior in the elevated plus maze test, pain sensitivity in the hot-plate test, and the fear responses in a contextual fear test on postnatal day 60. We also examined sex-specific effects of NI and NTS on these behavioral tests.

2. Materials and methods

2.1. Animals

Pregnant female Sprague–Dawley rats were purchased from Charles River (Yokohama, Japan). The rats were housed individually in the breeding colony at constant room temperature (23 ± 2 °C) and humidity (60%) with a 12/12 h light–dark cycle (lights on at 08:00). Food (Rodent Lab Diet EQ 5L37, Japan SLC Inc.) and water, conforming to the Water Quality Standard required by the Japanese Waterworks Law, were provided ad libitum. Male (n = 153) and female (n = 151) SD rats were used, and no more than two pups from the same dam were used in behavioral experiments. The experimental animals were divided into the following groups: (1) sham-treatment, (2) NI, and (3) NTS. Prior to birth, litters from each dam were randomly assigned to the sham, NI, and NTS groups. Litters were weaned on postnatal (PN) day 22. After weaning, male and female rats were housed in same-sex, same-treatment groups of three per cage (38 cm × 23 cm × 20 cm stainless steel cage) and maintained under normal conditions until the behavioral experiments; these included the open-field locomotor test (males; sham: n = 10, NI: n = 10, NTS: n = 10, females; sham: n = 10, NI: n = 10, NTS: n = 10), elevated plus maze test (males; sham: n = 12, NI: n = 16, NTS: n = 12, females; sham: n = 12, NI: n = 13, NTS: n = 15), hot-plate test (males; sham: n = 12, NI: n = 12, NTS: n = 15, females; sham: n = 12, NI: n = 11, NTS: n = 12), and contextual fear-conditioning test (males; sham: n = 14, NI: n = 14, NTS: n = 16, females; sham: n = 15, NI: n = 16, NTS: n = 15), which were undertaken on PN day 60 (Fig. 1). A different set of rats was used for each of these experiments. All animal procedures were approved by the Hiroshima University Medical Science Animal Care Committee.

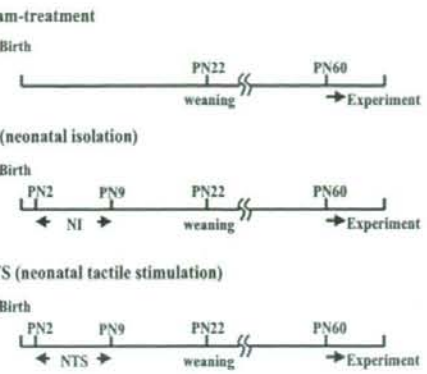

Fig. 1. Animal treatment paradigms. Prior to birth, litters from each dam were randomly assigned to (A) sham, (B) NI, and (C) NTS groups depending on neonatal treatments. All litters were weaned on postnatal (PN) day 22. After weaning, male and female rats in all groups were housed in same-sex, same-treatment groups of three per cage. Behavioral experiments were performed on PN day 60.

2.2. Neonatal isolation (NI)

After birth, the pups and mothers were housed together in their home cages (38 × 23 × 20 cm clear plastic cages) until weaning. Kehoe and Bronzino's method [9] was used for NI treatment. The first 24-h period after birth was designated PN day 1. Only litters with 11–14 pups were used in this study, and there were no differences in mean litter size among the three groups (NI, NTS, sham-treatment). The number of male and female pups was equal or almost equal in each litter (e.g., five males, six females). In the NI group, pups were isolated from the dam, nest, and siblings, and placed in individual opaque round containers (7 cm diameter and 8 cm deep) without bedding in a temperature- and humidity-controlled chamber, for 1 h per day on PN days 2–9. This microenvironment temperature was 30 ± 2 °C, similar to nest temperature, and humidity was 60%. Containers were placed 20 cm apart. Isolation was carried out between 09:00 and 12:00 each day. The rats in the sham group was housed under normal conditions and left undisturbed, except for weekly cage cleaning, until weaning. The rats in the sham group were similar to what are usually designated as animal facility-reared (AFR) animals.

2.3. Neonatal tactile stimulation (NTS)

Pups were isolated from the dam, nest, and siblings, and placed in individual round containers, as described above for NI. All pups were then gently handled dorsally from head to tail for 1 h per day by an investigator whose hands were covered with fine latex gloves. After handling, all pups were returned to the home cage at the same time. The duration of each handling session was approximately 30 s per pup and each pup was handled for a total of 5 min. This procedure was conducted on PN days 2–9.

2.4. Body weight

Body weight (g) was measured on the day of weaning (PN day 22), PN day 40, and on PN day 60.

2.5. Behavioral studies

All behavioral experiments were undertaken on PN day 60, and the animals were left without handling in the home cage (38 cm × 23 cm × 20 cm stainless steel cage), except for the measurement of body weight on PN day 40 until testing started. Rats were tested between 08:00 and 12:00. All behavioral data were collected by blind observers who were seated inside the testing room.

2.5.1. Open-field locomotor test

In the open-field locomotor test, rats were placed at the centre of a cubic chamber (48 cm × 48 cm × 48 cm). The animal's horizontal movements, measured by automatic actography (SCANET MV-10; Melquest, Toyama, Japan), were estimated as the number of interruptions of the near infrared rays. The infrared sensors were set at a height of 10 cm from the floor, and the distance between the photo beams was 6 mm. Additionally, the number of rearings (standing upright on the hind legs) was also measured. All animals were habituated to the testing room for 20 min before the start of each session and the testing sessions lasted for 5 min. The open-field area was cleaned with 10% ethanol and then with water between each experiment. The test room was dimly illuminated with indirect white lighting.

2.5.2. Elevated plus maze test

The plus maze consisted of two open (50 cm × 10 cm) and two closed (50 cm × 10 cm × 38 cm) arms, arranged perpendicularly, and was elevated 73 cm above the floor. Each rat was placed in the center of the apparatus and the number of entries and time spent per open and closed arms was recorded via a video camera mounted above the center of the apparatus. Each rat was habituated to the testing room for at least 20 min before being placed in the center of the maze. The apparatus was cleaned with alcohol after each rat was tested. The trial lasted for 5 min, after which the rat was removed from the maze and returned to its home cage. The test room was dimly illuminated with indirect white lighting. The duration of time spent in arms, the number of visits to arms, the percentage of time in the open arms (time in the open arms divided by the time in both arms), the percentage of open arm entries (number of entries into the open arm divided by total number of entries in both arms), total entries (the number of open plus closed arm entries), and head dippings from the open arm (protrusion of the head over the edge of an open arm) were evaluated.

2.5.3. Hot-plate test

The hot-plate test is one of the most commonly used methods for determining analgesic efficacy in rodents. Each rat was placed in a glass beaker on a hot-plate (HPT-1; Melquest, Toyama, Japan). A hot-plate analgesia meter, maintained at 52.5 °C, was used for this experiment. Latency to flinch or raise hind paws was recorded. To prevent tissue damage, the rat was removed from the hot plate if it did not respond within 30 s [34].

2.5.4. Contextual fear-conditioning test

Fear-conditioning tests were performed as follows: The conditioning chamber was located in a windowless room and housed in a soundproof box (70 cm × 60 cm × 60 cm). The conditioning chamber (50 cm × 28 cm × 32.5 cm) was made of transparent acrylic resin on three sides and aluminum on the other two. One of the metal sides had a speaker and three 24 V lights. A clear plexiglass window allowed the rat to be continually observed. The chamber was equipped with an 18-bar insulated shock grid floor. The floor was removable, and between tests the floor and interior of the chamber were cleaned with 70% ethanol and then with water. Each bar (5 mm in diameter) was connected through a harness to a shock generator-scrambler (Model SGS003; Muromachi, Tokyo, Japan), a device that delivers scrambled shock. Only one rat at a time was present in the experimental room. The other rats remained in their home cages. Each rat was carried to the behavioral room in a fresh cage that was identical to the home cage.

For the contextual conditioning experiments, rats were placed in the conditioning chamber 180 s before the onset of the unconditioned stimuli (US) (continuous foot shock at 0.8 mA for 4 s). After the test, rats were placed back in their home cages. Twenty-four hours later, rats were placed again in the same conditioning chamber and contextual freezing was assessed. Conditioning was assessed based on measurements of freezing, defined as the total absence of body and head movement, except for that associated with breathing. Freezing behavior of the rat was recorded using a video recorder for 180 s after the onset of the conditioned stimuli, and later scored blindly by the experimenter. Fear was quantified as the amount of time (in s) spent freezing.

2.6. Statistical analysis

Behavioral parameters were expressed as the mean (±S.E.M.). Statistical analysis was performed by two-way analysis of variance (ANOVA) to compare the means in different groups of each gender. One factor was neonatal treatment (sham-treatment or NI, or NTS), and another factor was gender (male or female). In case there were no gender differences, we analyzed treatment effects with pairing of gender on littermates. Tukey's test was used for multiple comparisons. Statistical significance between groups was set at $p < 0.05$.

3. Results

3.1. Body weight

Mean body weights of the groups on PN day 22 (weaning day), PN day 40, and PN day 60 are presented in Table 1. While two-way ANOVA revealed a significant effect of gender [$F(1, 144) = 8.799, p < 0.05$], [$F(1, 144) = 1300.440, p < 0.0001$], [$F(1, 144) = 2150.624, p < 0.001$], on PN days 22, 40, and 60, respectively, there was no significant effect of neonatal treatment nor an interaction between neonatal treatment and gender. On weaning day, the mean body weight of females was significantly greater than that of males. In contrast, the mean body weight of males was significantly greater than that of females in adulthood.

3.2. Open-field locomotor test

In the open-field locomotor test, two-way ANOVA revealed significant effects of neonatal treatment [$F(2, 54) = 16.353$, $p < 0.0001$], and gender [$F(1, 54) = 6.911, p < 0.05$] on horizontal movements. No significant interaction between neonatal treatment and gender [$F(2, 54) = 2.570$, $p = 0.186$] was found. Post hoc comparison revealed that the mean number of horizontal movements of rats in the NTS group was significantly higher

Table 1
Body weights (g) on the day of weaning (PN day 22), 40, and 60 of Sprague–Dawley (SD) male and female rats (sham, NI, NTS)

Group	Weaning (PN22)	PN40	PN60
Male			
Sham	51.4 ± 1.8	242.8 ± 1.9*	341.6 ± 3.7
NI	48.8 ± 2.3	242.1 ± 3.9*	337.8 ± 3.0[$]
NTS	52.8 ± 1.1	245.1 ± 2.4*	339.8 ± 3.8[$]
Female			
Sham	54.5 ± 1.6[#]	165.2 ± 2.8	205.1 ± 4.4
NI	53.7 ± 0.9[#]	164.4 ± 2.9	208.3 ± 2.6
NTS	55.8 ± 1.0[#]	166.4 ± 1.5	212.4 ± 2.9

Each group consisted of male ($n = 25$/group) and female ($n = 25$/group) rats. Values were expressed as the means ± S.E.M. On weaning day (PN 22), females exhibited significant greater body weight than males. Males demonstrated significant greater body weight than females on PN 40 and 60, [#]$p < 0.05$ compared with males (PN 22). *$p < 0.0001$ compared with females (PN 40). [$]$p < 0.001$ compared with females (PN 60).

Table 2
Effects of two different neonatal treatments on locomotor activity in adult rats

	Sham	NI	NTS
(a) Horizontal movements (counts/5 min)			
Male	2139.7 ± 106.7	2098.4 ± 103.2	2549.2 ± 150.2*
Female	2054.4 ± 94.8#	1602.3 ± 58.2#	2440.8 ± 134.4*,#
(b) Rearing			
Male	16.9 ± 2.1	12.0 ± 1.9$	27.8 ± 2.7$,&
Female	15.8 ± 0.8	10.2 ± 0.9$	24.6 ± 1.2$,&

The levels of horizontal locomotor activity (a), and rearing (b), among groups in the open-field locomotor test. Each group consisted of male ($n = 10$/group) and female ($n = 10$/group) rats. Data were expressed as the means ± S.E.M. *$p < 0.0001$ compared with the NI group. #$p < 0.05$ compared with males. $$p < 0.05$ compared with the sham group. &Indicates a significant difference ($p < 0.0001$) between the NI and the NTS groups.

than that of rats in the NI group. The mean number of horizontal movements of female rats was lower than that of male rats (Table 2a).

Two-way ANOVA revealed a significant effect of neonatal treatment on rearing [$F(2, 54) = 38.335$, $p < 0.0001$], but no significant of gender [$F(1, 54) = 2.655$, $p = 0.109$] and no significant interaction between neonatal treatment and gender [$F(2, 54) = 0.303$, $p = 0.740$]. Post hoc comparison showed that the mean rearing of the NTS group was significantly higher than that of the other two groups, and that was significantly lower in the NI group than in the sham group (Table 2b).

3.3. Elevated plus maze test

For the elevated plus maze test, two-way ANOVA revealed a significant effect of neonatal treatment [$F(2, 74) = 27.919$, $p < 0.0001$], but no significant effect of gender [$F(1, 74) = 1.604$, $p = 0.209$] and no significant interaction between neonatal treatment and gender [$F(2, 74) = 0.556$, $p = 0.576$] on the duration of time spent in open arms. Post hoc comparison showed that the duration of time spent in open arms in the NTS group was significantly longer than in the sham and NI groups and was significantly shorter in the NI group than in the sham group. Two-way ANOVA of data on the duration of time spent in closed arms revealed a significant effect of neonatal treatment [$F(2, 74) = 6.310$, $p < 0.001$] but no significant effect of gender [$F(1, 74) = 0.990$, $p = 0.323$] and no significant interaction between neonatal treatment and gender [$F(2, 74) = 0.453$, $p = 0.637$]. Post hoc comparison showed that the duration of time spent in closed arms in the NTS group was significantly shorter than in the NI group (Table 3).

Two-way ANOVA of data on the number of visits to open arms revealed significant effects of neonatal treatment [$F(2, 74) = 28.100$, $p < 0.0001$], and gender [$F(1, 74) = 10.250$, $p < 0.001$], but there was no significant interaction between these variables [$F(2, 74) = 0.665$, $p = 0.517$]. Post hoc comparison showed that the number of visits to open arms in the NTS group was significantly higher than in the sham and NI groups, and significantly lower in the NI group than in the sham group. The mean number of visits to open arms for female rats was lower than that for male rats. For the number of visits to closed arms, two-way ANOVA revealed a significant effect of gender [$F(1, 74) = 33.360$, $p < 0.0001$] but no significant of neonatal treatment [$F(2, 74) = 0.747$, $p = 0.477$] and no significant interaction between neonatal treatment and gender [$F(2, 74) = 3.728$, $p = 0.069$]. Post hoc comparison showed that the mean number of visits to closed arms for female rats was lower than that for male rats (Table 3).

On the percentage of time spent in the open arms (% open arm time), two-way ANOVA revealed a significant effect of neonatal treatment [$F(2, 74) = 24.550$, $p < 0.0001$] but no significant of gender [$F(1, 74) = 0.156$, $p = 0.694$] and no significant interaction between neonatal treatment and gender [$F(2, 74) = 0.589$, $p = 0.557$]. Post hoc comparison showed that the % open arm time in the NTS group was significantly higher than in the sham and NI groups. The % open arm time was significantly lower in the NI group than in the sham group. Similarly, two-way ANOVA revealed a significant effect of neonatal treatment [$F(2, 74) = 22.979$, $p < 0.0001$], no significant of gender [$F(1, 74) = 0.427$, $p = 0.515$] and no significant interaction between

Table 3
Effects of two different neonatal treatments on explorative and anxiety-like behavior in adult rats

	Male			Female		
	Sham	NI	NTS	Sham	NI	NTS
Duration of time spent in arms (s)						
Open arms	55.7 ± 4.1	48.4 ± 5.4*	102.4 ± 6.5*,#	77.4 ± 5.1	42.8 ± 4.4*	107.7 ± 8.4*,#
Closed arms	156.6 ± 6.1	169.1 ± 7.8	138.8 ± 9.6#	171.1 ± 8.8	186.7 ± 8.3	135.6 ± 9.4#
Number of visits spent in arms						
Open arms	8.9 ± 0.6	6.5 ± 0.5*	10.7 ± 1.0*,#	7.3 ± 0.8&	3.7 ± 0.5*,&	9.6 ± 0.6*,#,&
Closed arms	8.8 ± 0.5	8.3 ± 0.5	7.5 ± 0.4	5.7 ± 0.4&	5.1 ± 0.3&	6.7 ± 0.5&
%open arm time	26.2 ± 1.7	22.9 ± 2.8*	42.7 ± 4.6*,#	31.3 ± 3.2	18.1 ± 1.7*	43.9 ± 3.8*,#
%open arm entries	50.2 ± 3.3	42.9 ± 2.9*	57.8 ± 3.6*,#	54.8 ± 2.9	41.1 ± 3.7*	59.2 ± 2.8*,#
Total entries	17.7 ± 0.8$	14.8 ± 0.9	18.2 ± 1.4$	12.9 ± 1.2$,&	8.9 ± 1.1&	16.3 ± 0.9$,&
Head dippings	7.0 ± 0.6$	4.1 ± 0.4	10.5 ± 0.5$	6.8 ± 0.4$	4.8 ± 0.7	8.5 ± 0.4$

Comparison of the duration of time spent in arms, the number of visits to arms, the percentage of time in the open arms (%open arm time), the percentage of open arm entries (%open entries), total entries, head dippings from the open arm, among groups in the elevated plus maze test. Each group consisted of male (sham: $n = 12$, NI: $n = 16$, NTS: $n = 12$) and female (sham: $n = 12$, NI: $n = 13$, NTS: $n = 15$) rats. Data were expressed as the means ± S.E.M. *$p < 0.05$ compared with the sham group, #indicates a significant difference ($p < 0.0001$) between the NI and the NTS groups, $$p < 0.05$ compared with the NI groups, &$p < 0.05$ compared with males.

neonatal treatment and gender [$F(2, 74) = 0.824$, $p = 0.443$] on the percentage of entries in the open arms (% open arm entries) (Table 3).

Locomotion in the elevated plus maze was reflected by the total number of entries (total entries). While two-way ANOVA revealed significant effects of neonatal treatment [$F(2, 74) = 16.074$, $p < 0.0001$], and gender [$F(1, 74) = 27.119$, $p < 0.0001$], there was no significant interaction between these variables [$F(2, 74) = 2.309$, $p = 0.106$]. Post hoc comparison revealed that the mean number of total entries was significantly lower in the NI group than in the sham and NTS groups. The mean number of total entries for female rats was lower than that for male rats (Table 3).

Head dipping in the elevated plus maze test is an exploratory behavior. Two-way ANOVA revealed a significant effect of neonatal treatment [$F(2, 74) = 56.056$, $p < 0.0001$], no significant of gender [$F(1, 74) = 1.534$, $p = 0.219$], and a significant interaction between neonatal treatment and gender [$F(2, 74) = 3.849$, $p < 0.05$]. Post hoc comparison revealed that the mean number of head dippings was significantly lower in the NI group than in the sham and NTS groups (Table 3).

3.4. Hot-plate test

In the hot-plate test, two-way ANOVA revealed significant effects of neonatal treatment [$F(2, 68) = 44.326$, $p < 0.0001$], and gender [$F(1, 68) = 4.657$, $p < 0.05$] on latency to flinch or raise hind paws. No significant interaction between neonatal treatment and gender [$F(2, 68) = 1.270$, $p = 0.287$] was found. Post hoc analysis revealed that the latency was significantly longer in the NTS group than in the other two groups, and that the latency was significantly shorter in the NI group than in the sham group. The latency for female rats was shorter than that for male rats (Table 4).

3.5. Contextual fear-conditioning test

In the contextual fear-conditioning test, two-way ANOVA revealed significant effects of neonatal treatment [$F(2, 84) = 15.636$, $p < 0.0001$] and gender [$F(1, 84) = 9.934$, $p < 0.01$], and a significant interaction between neonatal treatment and gender [$F(2, 84) = 6.710$, $p < 0.01$] on contextual freezing time. Post hoc comparison revealed that the contextual freezing time was significantly lower in NTS males than in sham males. The contextual freezing time of NI males tended to be significantly lower than that of sham males ($p = 0.09$).

Table 4
Effects of two different neonatal treatments on pain sensitivity in adult rats

	Sham	NI	NTS
Male	12.5 ± 1.3	11.0 ± 0.7*	15.6 ± 2.6*,#
Female	10.8 ± 1.8$	9.5 ± 0.9*,$	15.7 ± 2.2*,#

Comparison of latency (s) to flinch or raise hind paws among groups in the hot-plate test. Each group consisted of male (sham: $n = 12$, NI: $n = 12$, NTS: $n = 15$) and female (sham: $n = 12$, NI: $n = 11$, NTS: $n = 12$) rats. Data were expressed as the means ± S.E.M. *$p < 0.05$ compared with the sham group, #$p < 0.05$ compared with the NI group, $$p < 0.05$ compared with males.

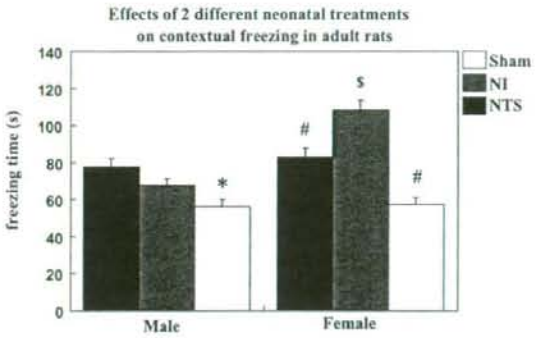

Fig. 2. Effects of two different neonatal treatments on contextual freezing time (s) in adult rats during a 180-s interval. Each group consisted of male (sham: $n = 14$, NI: $n = 14$, NTS: $n = 16$) and female (sham: $n = 15$, NI: $n = 16$, NTS: $n = 15$) rats. The results were expressed as the mean ± S.E.M. (*) indicates a significant difference ($p < 0.05$) between sham males and NTS males; (#) $p < 0.05$ compared with NI females; ($) indicates a significant difference ($p < 0.0001$) between NI males and NI females.

The enhancement of contextual freezing time was significantly greater in NI females than in sham or NTS females. NTS females showed significantly less contextual freezing than sham females. Surprisingly, contextual freezing time was enhanced more in NI females than in NI males (Fig. 2).

4. Discussion

In the present study, we examined how two different types of neonatal manipulations (NI as an adverse experience versus NTS as an intervention for adverse experience) in rats affect development, locomotor and exploratory behavior, anxiety-like behavior, pain sensitivity, and contextual fear in adulthood. Furthermore, we examined the sex-specific effect of neonatal manipulations on the susceptibility to environmental stimuli in adulthood. This study had three major findings: (1) Neither NI nor NTS affects gross physical development. (2) Among both sexes, NTS can reverse the decrease in rearing in the open-field locomotor test, the enhanced anxiety-like behavior in the elevated plus maze test, and the increased pain sensitivity in the hot-plate test of rats subjected to NI in adulthood. In most of these behavioral tests, NTS can promote behavioral and emotional responses compared to rats subjected to sham-treatment. The rats subjected to NTS showed increased rearing, decreased anxiety-like behavior, pain sensitivity, and contextual freezing to compare with those subjected to sham-treatment. (3) In the contextual fear-conditioning test in adulthood, there is a significant interaction between neonatal treatments and gender. NTS can reverse the enhanced fear induced by NI in female but not male rats. Overall, the results of the present study of NI indicated that this neonatal treatment reduced rearing and explorative behaviors, and enhanced anxiety-like behavior among both sexes. These findings are in agreement with a previous report demonstrating that maternally separated rats showed higher levels of anxiety and fear than sham-treated rats in adulthood, as measured by the open-field test and elevated plus maze test [8].

Furthermore, our findings apparently showed that NTS induced compensatory as well as enhancing effects on the activities and emotional states of both sexes.

Pauk et al. [25] previously demonstrated that tactile stimulation with a camel hair brush during maternal deprivation for 2 h prevented the increase of serum corticosterone in response to maternal deprivation in infants rats, which is similar to the effect of NTS observed in the present study. Although further studies are needed to verify the efficacy of tactile stimulation, it is postulated that neonatal tactile stimulation may prevent the developmental disturbance of emotionality in response to early adversity. On the other hand, it is well known that maternal behavior plays an important role in physical and psychological development through mother–infant contact. For example, early adversity such as NI [3] or low maternal care [6] was reported to enhance activity of the HPA axis in response to stress in later life. In this context, it cannot be ruled out that the ability of NTS to reverse the effect of NI in these behavioral tests may be due to differences in maternal care for 23 h/day between the NI and NTS groups, since we did not measure maternal behaviors such as licking and grooming or arched-back nursing in this study.

In the contextual fear-conditioning test, whereas NTS led to a significant reduction in contextual freezing among both sexes as compared with sham-treatment, NTS reversed the NI-induced increase in contextual freezing in female but not male rats. The differential effect of NTS on contextual freezing in male and female rats may be due to differences in the effect of NI, since NI significantly enhanced contextual freezing in female but not male rats. The results of the present study are almost consistent with a previous study which showed that 1 h-neonatal isolation on PN days 2–9 enhances contextual fear in female Sprague–Dawley rats and impairs it in male Sprague–Dawley rats [11]. It has been suggested that contextual freezing behavior is fear and anxiety-related response, at least in part, mediated by the hippocampus [5,26]. We speculated, based on the results of these studies, that the intensity of hippocampal-dependent fear and anxiety responses in adulthood due to early adversity may differ between male and female rats.

There was no significant interaction between neonatal treatments and gender with respect to locomotor activities or rearing movements in the open-field test, anxiety-like behavior without head dippings in the elevated plus maze test, or pain sensitivity in the hot-plate test. In contrast, Rhees et al. [28] reported that females were more active than males in both the sham-treated and maternally separated groups. This difference may be due to differences in the experimental paradigms such as the breeding environment, age of animals, period of handling, etc.

It is noteworthy that the estrous cycle is an important factor for mediating stress responses in females. Unfortunately, in the present experiments, the behaviors of the female rats were not tested at any specified or consistent time in their estrous cycle. Rat behavior in an open-field test was reported to be independent of hormonal variations during the estrous cycle [33], and no significant difference in anxiety-like behavior was seen in the elevated plus maze test during proestrus and diestrus [23]. In contrast, female rats were reported to show higher % open arm times and % open arm entries during proestrus and estrus than during diestrus [22]. Furthermore, Severino reported that there was no significant interaction between neonatal handling and estrous cycle phases on the percentage of open arm time and percentage of open arm entries, but not total entries [31]. They reported that in diestrus the total number of arm entries in handled females was higher than in the nonhandled group in the same phase and also higher than in the handled females in estrus. Based on these findings, further studies examining the influence of the estrous cycle on anxiety-like behavior in the open arm test are required.

There are several limitations in this study that should be taken into consideration. First, the behavioral experiments in adulthood were undertaken during the light period but not the dark period of the dark/light cycle. As in our study, numerous studies examining the influence of neonatal manipulations on the adulthood behavioral responses to environmental stimuli, were performed during the light phase [2,13,24,28,30–32]. However, since the differences in the locomotor activity or anxiety-like behavior [7,8] between the light and dark phase were reported, assessment during both the light and dark phase is required to promote our understanding of behavioral responses. Secondly, the behaviors of the female rats were not tested at any specified or consistent time in their estrous cycle. Thirdly, although Kosten et al. [13] showed that 1 h of neonatal isolation during PN days 2–9 did not affect on estrous stage cyclicity, it cannot be ruled out that the neonatal handling may affect estrous stage cyclicity and may subsequently change the neuromodulatory role of gonadal steroids on the adulthood behavior.

In summary, the present study clearly indicated that whereas NI altered behavioral and emotional responses to environmental stimuli in later life, NTS could reverse the effect of NI and promote behavioral and emotional responses, to the same extent, in both sexes. In other words, an adequate tactile stimulation in early life plays an important role in the prevention of susceptibility to environmental stimuli induced by an early adverse experience. In addition, long-lasting behavioral and emotional changes, induced by an early adverse experience, differ somewhat between male and female rats in adulthood.

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References

[1] Beane ML, Cole MA, Spencer RL, Rudy JW. Neonatal handling enhances contextual fear conditioning and alters corticosterone stress responses in young rats. Horm Behav 2002;41:33–40.

[2] Cannizzaro C, Martire M, Cannizzaro E, Provenzano G, Gagliano M, Carollo A, et al. Long-lasting handling affects behavioural reactivity in adult rats of both sexes prenatally exposed to diazepam. Brain Res 2001;22:225–33.

[3] Erabi K, Morinobu S, Kawano KI, Tsuji S, Yamawaki S. Neonatal isolation changes the expression of IGF-IR and IGFBP-2 in the hippocampus in response to adulthood restraint stress. Int J Neuropsychopharmacol 2006:1–13.
[4] Erskine MS, Stern JM, Levine S. Effects of prepubertal handling on shock-induced fighting and ACTH in male and female rats. Physiol Behav 1975;14:413–20.
[5] Fanselow MS, Helmstetter FJ. Conditional analgesia, defensive freezing, and benzodiazepines. Behav Neurosci 1988;102:233–43.
[6] Fish EW, Shahrokh D, Bagot R, Caldji C, Bredy T, Szyf M, et al. Epigenetic programming of stress responses through variations in maternal care. Ann N Y Acad Sci 2004;1036:167–80.
[7] Jones N, King SM. Influence of circadian phase and test illumination on pre-clinical models of anxiety. Physiol Behav 2001;72:99–106.
[8] Kalinichev M, Easterling KW, Plotsky PM, Holtzman SG. Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. Pharmacol Biochem Behav 2002;73:131–40.
[9] Kehoe P, Bronzino JD. Neonatal stress alters LTP in freely moving male and female adult rats. Hippocampus 1999;9:651–8.
[10] Knuth ED, Etgen AM. Long-term behavioral consequences of brief, repeated neonatal isolation. Brain Res 2007;1128:139–47.
[11] Kosten TA, Miserendino MJ, Bombace JC, Lee HJ, Kim JJ. Sex-selective effects of neonatal isolation on fear conditioning and foot shock sensitivity. Behav Brain Res 2005;157:235–44.
[12] Kosten TA, Miserendino MJ, Kehoe P. Enhanced acquisition of cocaine self-administration in adult rats with neonatal isolation stress experience. Brain Res 2000;875:44–50.
[13] Kosten TA, Sanchez H, Jatlow PI, Kehoe P. Neonatal isolation alters the estrous cycle interactions on the acute behavioral effects of cocaine. Psychoneuroendocrinology 2005;30:753–61.
[14] Kosten TA, Sanchez H, Zhang XY, Kehoe P. Neonatal isolation enhances acquisition of cocaine self-administration and food responding in female rats. Behav Brain Res 2004;151:137–49.
[15] Levine S. Infantile experience and resistance to physiological stress. Science 1957;126:405.
[16] Levine S, Otis LS. The effects of handling before and after weaning on the resistance of albino rats to later deprivation. Can J Psychol 1958;12: 103–8.
[17] Liu D, Caldji C, Sharma S, Plotsky PM, Meaney MJ. Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinepherine release in the hypothalamic paraventricular nucleus. J Neuroendocrinol 2000;12:5–12.
[18] Liu D, Diorio J, Day JC, Francis DD, Meaney MJ. Maternal care, hippocampal synaptogenesis and cognitive development in rats. Nat Neurosci 2000;3:799–806.
[19] McIntosh J, Anisman H, Merali Z. Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender-dependent effects. Brain Res Dev Brain Res 1999;113:97–106.
[20] Meaney MJ. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci 2001;24:1161–92.
[21] Meaney MJ, Aitken DH, Viau V, Sharma S, Sarrieau A. Neonatal handling alters adrenocortical negative feedback sensitivity and hippocampal type II glucocorticoid receptor binding in the rat. Neuroendocrinology 1989;50:597–604.
[22] Mora S, Dussaubat N, Diaz-Veliz G. Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. Psychoneuroendocrinology 1996;21:609–20.
[23] Nomikos GG, Spyraki C. Influence of oestrogen on spontaneous and diazepam-induced exploration of rats in an elevated plus maze. Neuropharmacology 1988;27:691–6.
[24] Papaioannou A, Gerozissis K, Prokopiou A, Bolaris S, Stylianopoulou F. Sex differences in the effects of neonatal handling on the animal's response to stress and the vulnerability for depressive behaviour. Behav Brain Res 2002;129:131–9.
[25] Pauk J, Kuhn CM, Field TM, Schanberg SM. Positive effects of tactile versus kinesthetic or vestibular stimulation on neuroendocrine and ODC activity in maternally-deprived rat pups. Life Sci 1986;39:2081–7.
[26] Phillips RG, LeDoux JE. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci 1992;106:274–85.
[27] Ploj K, Pham TM, Bergstrom L, Mohammed AH, Henriksson BG, Nylander I. Neonatal handling in rats induces long-term effects on dynorphin peptides. Neuropeptides 1999;33:468–74.
[28] Rhees RW, Lephart ED, Eliason D. Effects of maternal separation during early postnatal development on male sexual behavior and female reproductive function. Behav Brain Res 2001;123:1–10.
[29] Rodrigues AL, Arteni NS, Abel C, Zylbersztejn D, Chazan R, Viola G, et al. Tactile stimulation and maternal separation prevent hippocampal damage in rats submitted to neonatal hypoxia–ischemia. Brain Res 2004;1002:94–9.
[30] Romeo RD, Mueller A, Sisti HM, Ogawa S, McEwen BS, Brake WG. Anxiety and fear behaviors in adult male and female C57BL/6 mice are modulated by maternal separation. Horm Behav 2003;43:561–7.
[31] Severino GS, Fossati IA, Padoin MJ, Gomes CM, Trevizan L, Sanvitto GL, et al. Effects of neonatal handling on the behavior and prolactin stress response in male and female rats at various ages and estrous cycle phases of females. Physiol Behav 2004;81:489–98.
[32] Silveira PP, Portella AK, Clemente Z, Gamaro GD, Dalmaz C. The effect of neonatal handling on adult feeding behavior is not an anxiety-like behavior. Int J Dev Neurosci 2005;23:93–9.
[33] Steiner M, Katz RJ, Carroll BJ. Behavioral effects of dopamine agonists across the estrous cycle in rats. Psychopharmacology (Berl) 1980;71:147–51.
[34] Stephan M, Helfritz F, Pabst R, von Horsten S. Postnatally induced differences in adult pain sensitivity depend on genetics, gender and specific experiences: reversal of maternal deprivation effects by additional postnatal tactile stimulation or chronic imipramine treatment. Behav Brain Res 2002;133:149–58.
[35] Stephan M, Straub RH, Breivik T, Pabst R, von Horsten S. Postnatal maternal deprivation aggravates experimental autoimmune encephalomyelitis in adult Lewis rats: reversal by chronic imipramine treatment. Int J Dev Neurosci 2002;20:125–32.
[36] Weinberg J, Levine S. Early handling influences on behavioral and physiological responses during active avoidance. Dev Psychobiol 1977;10:161–9.
[37] Wigger A, Neumann ID. Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. Physiol Behav 1999;66:293–302.

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Addition of risperidone to sertraline improves sertraline-resistant refractory depression without influencing plasma concentrations of sertraline and desmethylsertraline

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In the present study, we examined the efficacy of risperidone addition on sertraline-resistant depressed patients and the effects of risperidone on the metabolism of sertraline. Ten patients (M/F: 4/6, age: 54 ± 10 years) met the DSM-IV criteria for major depressive disorder enrolled the study. Hamilton Dating Scale for Depression (HAM-D) scores (mean ± SD) in all 10 patients significantly decreased from 19 ± 4 (before risperidone addition) to 11 ± 3 (4 weeks after risperidone addition). Plasma levels of sertraline and desmethylsertraline did not change after risperidone addition. Serum BDNF levels in responders to risperidone addition were changed from 8.1 ± 2.7 ng/ml (before risperidone addition) to 11.5 ± 0.9 ng/ml (4 weeks after risperidone addition); in contrast, those in nonresponders changed from 7.8 ± 2.2 ng/ml (before risperidone addition) to 7.9 ± 2.4 ng/ml (4 weeks after risperidone addition). These results suggest that the addition of risperidone to sertraline is effective and well tolerated for sertraline-resistant depressive patients, which is accompanied with the increase in serum BDNF levels in responders to the risperidone addition, and the addition of risperidone to sertraline does not seem to influence sertraline metabolism. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS — refractory depression; sertraline; desmethylsertraline; risperidone; BDNF

INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) and serotonin noradrenaline reuptake inhibitors (SNRIs) are generally considered first-line treatments for depression. Despite a sufficient dose and duration of antidepressant treatment 30–40% of all depressive patients fail to respond (Trivedi *et al.*, 2006). Further approaches in the treatment of resistant depression may be to switch to another drug of the same class or to another class of antidepressants (Kelsey, 1997), or to augment the antidepressant with a further drug such as thyroid hormones (Joffe, 1998), lithium (Roullion and Gorwood, 1998), bupropion (Bodkin *et al.*, 1997), pindolol (Blier and Bergeron, 1998), or dexamethasone (Dinan *et al.*, 1997).

Risperidone, a benzisoxazol derivative, belongs to the group of atypical antipsychotics. *In vitro* and *in vivo* studies have demonstrated that risperidone is a mixed serotonin ($5\text{-}HT_2$) and dopamine (D_2) receptor antagonist (Janssen *et al.*, 1988; Leysen *et al.*, 1988). We have reported that risperidone is effective for treating the acute phase of schizophrenia with few extrapyramidal side effects (Yoshimura *et al.*, 2003, 2005). Furthermore, we have reported that risperidone is also effective in treating negative symptoms of chronic schizophrenic patients (Yoshimura *et al.*, 2000a). In negative schizophrenic symptomatology and in mood disorders, a disturbance of the serotonergic system has been shown to be of relevance. Antiserotonergic drugs are used in the treatment of depression. In addition, various interactions between the dopaminergic and the serotonergic systems, indicating the complex nature of the serotonergic system, have been described (Marsden, 1991). Furthermore, our previous findings

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indicate that the effects of risperidone on the noradrenergic system as well as on the serotonergic system might be related to its efficacy in treating negative symptoms of schizophrenia (Yoshimura *et al.*, 2000a, 2000b) and mood disorder (Yoshimura *et al.*, 2006). Taken together, the above results show that the effects of risperidone on three monoaminergic systems (dopamine, serotonin, noradrenaline) are associated with its clinical efficacy for schizophrenia and mood disorder.

It has been reported that risperidone is increasingly being used to control acute manic episodes, and data are emerging to support its mood—stabilizing and antidepressant properties (Kasper *et al.*, 2002; Yatham, 2002). Furthermore, recent reports suggest that risperidone is useful for treating refractory depression (Goto *et al.*, 2005; Papakostas *et al.*, 2007). Rapaport *et al.* (2006) investigated the long-term efficacy of risperidone augmentation of SSRI treatment for resistant depression. In 57 in- and outpatient centers in three countries, the authors conducted a three-phase study with 4–6 weeks of open-label citaloplam monotherapy, 4–6 weeks of open-label risperidone augmentation, and a 24-week double-blind, placebo-controlled discontinuation phase. A total of 489 patients with major depressive disorder and 1–3 documented treatment failure entered the citalopram monotherapy phase (20–60 mg/day). Patients with <50% reduction in Hamilton Rating Scale for Depression (HAM-D) scores entered the risperidone augmentation phase (0.25–2.0 mg/day). Patients with HAM-D $\leqq 7$ or CGI-S $\leqq 2$ were randomized to risperidone or placebo augmentation. Of the 386 nonresponders who entered the augmentation phase, 243 remitted and 241 entered the double-blind phase. Median time to relapse was 102 days with risperidone augmentation and 85 days with placebo; relapse rates were 53.3 and 54.6%, respectively. These results suggest that open-label risperidone augmentation substantially enhanced response in treatment-resistant patients, but the longer-term benefits of augmentation were not demonstrated in the study.

In the present study, we investigated the efficacy of risperidone addition on sertraline-resistant refractory depressed patients, and we also examined the effects of risperidone on the metabolism of sertraline.

SUBJECTS AND METHODS

Ten patients met the DSM-IV criteria (American Psychiatric Association, 1994) for major depressive disorder enrolled the study. Four were male and six were female, and their ages ranged from 38 to 71 (mean $\pm$ SD $= 54 \pm 10$) years. All patients were physically healthy and free of current alcohol and/or drug abuse or comorbid anxiety or personality disorders. Drug resistance was defined as a failure to respond at least three course of a single antidepressant medication with adequate dose and duration (stage III definition from Thase and Rush, 1997). The protocol of this study was approved by the Ethics Committee of the University of Occupational and Environmental Health. All patients gave their consent to participate after having been informed of the study's purpose. The patients were treated with risperidone at doses ranging from 0.5 to 2.0 (mean $\pm$ SD $= 1.0 \pm 0.3$) mg/day. All patients were treated with sertraline at doses ranging from 50 to 100 (mean $\pm$ SD $= 87.5 \pm 16.7$) mg/day for at least 8 weeks, but their scores on the HAM-D (Hamilton, 1960) were 15 points or more and <50% reduction. The dosage of sertraline is considered to be low however upper limit of sertraline approved in Japan is 100 mg/day. Therefore, mean dosage of sertraline prescribed in the study was relatively high in Japanese standard. Risperidone was added to the ongoing sertraline. Only benzodiazepines were permitted as hypnotics, and the dosage was kept constant throughout the study period. The dosages of risperidone and sertraline varied among patients and, based on ethical considerations, were not fixed. However, doses of sertraline and risperidone were not altered during the co-medication period. The patients were evaluated regarding their clinical improvement and extrapyramidal symptoms using the HAM-D and Simpson and Angus Rating Scale (SAS) (Simpson and Angus, 1970), respectively, by two experienced psychiatrists (R.Y. and N.U.) The HAM-D was evaluated at before starting antidepressants (primary), before and every week after risperidone administration, and the SAS was evaluated before and every week after risperidone administration. Furthermore, blood levels of triglyceride and glucose and body weight were assessed before and 4 weeks after risperidone administration. The psychiatrists assessing the HAM-D and SAS were blind to the results of plasma levels of sertraline and desmethylsertraline or serum BDNF levels. Blood samples drawn into heparinized tubes were obtained at 08.00–10.00 before breakfast (approximately 13–15 h after the last dose of the drugs) before and at 4 weeks after risperidone treatment. The plasma samples were quickly separated in a centrifuge and stored at −80°C until assayed. The plasma levels of sertraline and desmethylsertraline were analyzed by high-performance liquid chromatography (HPLC) accord-

ing to the method of Mandrioli *et al.* (2006). In short, solid-phase extraction was carried out using Varian BondElut C2 cartridges (100 mg, 1 ml) on a Varian VacElut apparatus. Aliquots of 50 μl of internal standard (clomipramine) were added to 250 μl of patient's plasma. The resulting mixture was diluted with 500 μl of water and loaded onto a previously conditioning C2 cartridge. The cartridges were equilibrated five times with 1 ml of methanol and then conditioned five times with 1 ml of water. After loading, the cartridges were washed twice with 1 ml of water, then with 1 ml of methanol/10 mM, pH 10 carbonate buffer (20:80, v/v), and finally with 50 μl of methanol. Elution was carried out with 1 ml of methanol. The elute was brought to dryness (rotary evaporator), redissolved in 250 μl of mobile phase (acetonitrile and a 12.3 mM, pH 3 phosphate buffer containing 0.1% triethylamine 35:65, v/v), and injected into the HPLC system.

Plasma risperidone and 9-hydroxyrisperidone were analyzed using HPLC according to Olesen and Linnet (1997). In brief, 1 ml plasma was mixed with 0.5 ml 0.6 M sodium carbonate/bicarbonate buffer (pH = 10) and 50 μl of haloperidol solution, with 3.76 mg/ml (10 mM) as an internal standard. Next 8 ml heptane-isoamyl alcohol (98:2, v/v) was added, and the mixture was shaken for 5 min in the horizontal position at 250 shakes/min. After centrifugation at 1500*g* for 10 min, the aqueous layer was frozen by immersing the tubes in a cooling bath containing dry ice and ethanol. The heptane layer was decanted into centrifuge tubes and evaporated to dryness at 60°C in a gentle stream of nitrogen. The residue was dissolved in 75 μl mobile phase (40 mM ammonium acetate buffer pH 7—methanol; 100:900, v/v), of which 65 μl was injected into the HPLC.

The serum BDNF levels were measured using a BDNF Emax Immunoassay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. In short, 96-well microplates were coated with anti-BDNF monoclonal antibody and incubated at 4°C for 18 h. The plates were incubated in a blocking buffer for 1 h at room temperature. The samples were diluted with assay buffer 100-times and the BDNF standards were kept at room temperature under conditions of horizontal shaking for 2 h, followed by washing with the appropriate washing buffer. The plates were incubated with antihuman BDNF polyclonal antibody at room temperature for 2 h and washed with the washing buffer. The plates were then incubated with anti-IgY antibody conjugated to horseradish peroxidase for 1 h at room temperature, and incubated in peroxidase substrate and tetramethylbenzidine solution to induce a color reaction. The reaction was stopped with 1 mol/L hydrochloric acid. The absorbance at 450 nm was measured with an Emax automated microplates reader. The standard curve was linear from 5 to 5000 pg/ml, and the detection limit was 5 pg/ml. The intra- and interassay coefficients of variation were 5 and 7%, respectively. The recovery rate of the exogenously added BDNF in the measured plasma samples exceeded 95%.

Statistical analysis

Statistical analyses were conducted by the Wilcoxon test to compare HAM-D scores, plasma levels of sertraline, desmethylsertraline, triglyceride, and glucose, serum BDNF levels, or body weight before and 4 weeks after risperidone addition. The level of significance for all analysis was set at $p < 0.05$.

RESULTS

All participants completed the study (completion rate was 100%). HAM-D scores (mean ± SD) in all 10 patients were 22 ± 6, 19 ± 4, and 11 ± 3 at before starting sertraline (primary), before risperidone addition, 4 weeks after risperidone addition, respectively. HAM-D scores in all 10 patients significantly decreased 4 weeks after risperidone addition ($z = -2.023$, $p = 0.005$) (Figure 1). Five of 10 (50%) became responders (50% or more improvement in HAM-D scores) and 1 of 10 patients (10%) became remission (HAM-D scores of under seven points) in 4 weeks after risperidone addition. Plasma levels of sertraline and desmethylsertraline did not change after risperidone addition (Table 1). Furthermore, the

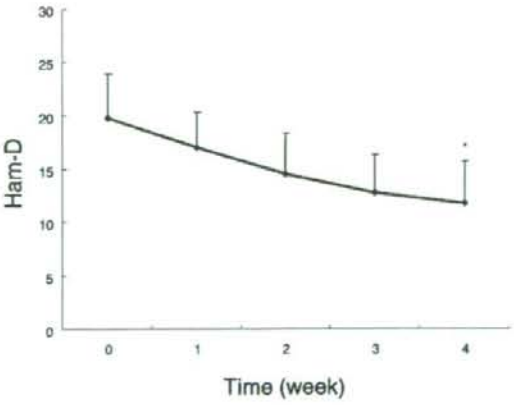

Figure 1. Changes in HAM-D scores after risperidone addition

Table 1. Plasma levels of sertraline and desmethylsertraline for each case

Case	Dose of ser (mg/day)	Dose of ris (mg/day)	Plasma ser level (B) (ng/ml)	Plasma dser level (B) (ng/ml)	Plasma ser level (4W) (ng/ml)	Plasma dser level (4W) (ng/ml)
1	100	0.5	44	49	39	35
2	100	1	58	62	50	51
3	75	0.5	39	35	48	44
4	100	1	51	45	44	49
5	50	1	16	16	23	20
6	75	1	22	31	14	20
7	100	2	39	42	33	35
8	75	1	29	32	33	30
9	100	1	49	53	52	55
10	100	1	61	58	54	59
Mean ± SD	87.5 ± 16.7	1.0 ± 0.38	40.8 ± 14.1	42.3 ± 13.3	39.0 ± 13.3	39.8 ± 13.0

ser, sertraline; dser, desmethylsertraline; rs, risperidone; B, before risperidone addition; 4W, 4 weeks after risperidone addition.

plasma level of active moiety of risperidone (risperidone plus 9-hydroxyrisperidone) at 4 weeks after risperidone administration was 17.7 ± 5.0 ng/ml. Serum BDNF levels in all patients slightly increased 4 weeks after risperidone addition (before risperidone addition: 7.9 ± 2.3 ng/ml, 4 weeks after risperidone addition: 9.7 ± 2.5 ng/ml), however the increase was not statistically significant ($z = -1.580$, $p = 0.1141$). Serum BDNF levels in responders to risperidone addition were changed from 8.1 ± 2.7 ng/ml (before risperidone addition) to 11.5 ± 0.9 ng/ml (4 weeks after risperidone addition) ($z = -2.023$, $p = 0.0431$); in contrast, those in nonresponders changed from 7.8 ± 2.2 ng/ml (before risperidone addition) to 7.9 ± 2.4 ng/ml (4 weeks after risperidone addition) (Figure 2). The SAS scores were not significantly different between before and 4 weeks after risperidone addition (before: 0.2 ± 0.4, 4 weeks after risperidone addition: 0.4 ± 0.4). In addition, blood levels in triglyceride and glucose and body weight were not significantly changed 4 weeks after risperidone addition.

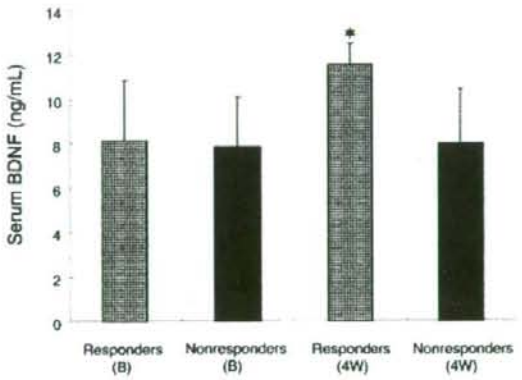

Figure 2. Effects of risperidone addition to sertraline on serum BDNF: B, before risperidone addition to sertraline; 4W, 4 weeks after risperidone addition to sertraline; responders, patients whose HAM-D scores decreased 50% or more; nonresponders, patients whose HAM-D scores decreased below 50%; $^{*}p = 0.043$, compared with B

DISCUSSION

Addition of a low dose of risperidone to sertraline was effective and well tolerated for patients with sertraline-resistant refractory depression. We have previously demonstrated that risperidone addition to antidepressants, such as SSRIs and tricyclic antidepressants, is effective for the treatment of psychotic depression, a subtype of refractory depression. In that study, we also found that plasma levels of homovanillic acid (HVA), a major metabolite of dopamine, in responders are higher than those in nonresponders. Furthermore, there is a negative correlation between changes in plasma HVA levels and per cent improvement on HAM-D scores in patients with psychotic depression. Taken together, the findings suggest that the influence of risperidone on the dopaminergic system might play an important role in its efficacy in psychotic depression. Rapaport *et al.* (2006) investigated the long-term efficacy of risperidone augmentation of SSRI treatment for resistant depression. In 57 in- and outpatient centers in three countries, the authors conducted a three-phase study with 4–6 weeks of open-label citaloplam monotherapy, 4–6 weeks of open-label risperidone augmentation, and a 24-week double-blind, placebo-controlled discontinuation phase. A total of 489 patients with major depressive disorder and 1–3 documented treatment failure entered the citalopram monotherapy phase. Patients

with <50% reduction in HAM-D scores entered the risperidone augmentation phase (0.25–2.0 mg/day). Patients with HAM-D ≤ 7 or CGI ≤ 2 were randomized to risperidone or placebo augmentation. The primary outcome was time to relapse during the double-blind phase. During citalopram therapy, 434 patients had <50% HAM-D reduction; 299 (68.9%) were fully nonresponsive and 135 were partially nonresponsive. Of the 386 nonresponders who entered the augmentation phase, 243 remitted and 241 entered the double-blind phase. Median time to relapse was 102 days with risperidone augmentation and 85 days with placebo. Mahmound *et al.* (2007) have reported that risperidone augmentation was associated with improvements in clinician-rated depressive symptoms and patients' perception of those symptoms, reduced disability, and increased response and remission rate in patients with suboptimal responses to standard antidepressant monotherapy. The response rate in the present study was 50%, which is a little higher than that of Mahmund's study (35.6%). The reason for this discrepancy might be related to the study design. Our study was an open-labeled, while that of Mahmund *et al.* was a randomized controlled study. In any cases, further studies should be needed to confirm the benefits of risperidone augmentation to antidepressants in refractory depression. A recent meta-analysis by Papakostas *et al.* (2007) demonstrated that augmentation of antidepressant with atypical antipsychotic drugs including olanzapine, risperidone, and quetiapine, for treatment-resistant depression is definitely effective based on both the response rate and remission rate.

Another important finding in the present study was that risperidone addition did not influence plasma levels of sertraline and its metabolite, desmethylsertraline. The major metabolic pathway of sertraline is the demethylation to form desmethylsertraline, a reaction primarily mediated by cytochrome P450 (cyp) 3A4. However, other cyp enzymes, including cyp2D6, 2C9, and 2C19, also play a significant role in mediating demethylation (Greenblatt *et al.*, 1999; Wang *et al.*, 2001). In contrast, risperidone is extensively metabolized primarily via 9-hydroxylation, yielding an active metabolite 9-hydroxyrisperidone. *In vivo* and *in vitro* studies have indicated that cyp 2D6 and, to a lesser extent cyp3A4 are involved in the 9-hydroxylation of risperidone. Therefore, it is plausible that the addition of risperidone to sertraline alters metabolization from sertraline to desmethylsertraline. Indeed, Wang *et al.* (2006) recently reported that risperidone administration to sertraline slightly shortened the absorption T1/2 and slightly decreased the Tmax of sertraline in plasma compared to sertraline administration alone. In the brain, risperidone coadministration greatly increased the Tmax of sertraline and remarkably shortened the T1/2 in brain. The authors proposed that these findings suggest that sertraline and/or desmethylsertraline interacted at sites of the blood brain barrier to increase the brain entry of risperidone and 9-hydroxyrisperidone. Both risperidone and 9-hydroxyrisperidone are substrates of p-glycoprotein. Therefore, the increment of brain risperidone and 9-hydroxyrisperidone by coadministered sertraline suggests an inhibitory effect on p-glycoprotein in blood brain barrier by sertraline and/or desmethylsertraline. It is possible that risperidone, as an inhibitor of p-glycoprotein, could decrease the efflux of sertraline and its metabolite out of the brain by p-glycoprotein inhibition thereby increasing the central nervous system accumulation of antidepressant leading to greater therapeutic effects (Wang *et al.*, 2006). As this interaction occurs at the level of the blood brain barrier, it is not necessarily detectable by measuring plasma drug concentrations alone.

However, the addition of risperidone to sertraline did not influence the metabolism of sertraline. The dose of risperidone was small, possibly because cyp2D6 might not play a major role in sertraline metabolism, or because the inhibitory effects of risperidone on cyp2D6 might not be potent. The average plasma sertraline levels in all patients were approximately 40 ng/ml before risperidone addition. Furthermore, no difference was found in plasma sertraline levels between the responders (37.0 ± 10.3 ng/ml) and the nonresponders (44.6 ± 16.2 ng/ml). Desmethylsertraline, an active metabolite of sertraline is up to 25 times weaker than sertraline *in vitro*, producing only with parent drug, desmethylsertraline being considered substantially less active than the parent compound playing a negligible role in its clinical activity. Although the biological activity in desmethylsertraline does not seem to be associated with the clinical efficacy of sertraline, plasma desmethylsertraline level also did not differ between the responders (39.8 ± 7.1 ng/ml) and the nonresponders (44.8 ± 17.1 ng/ml). Some studies have found that therapeutic drug monitoring may be useful for dose optimization as serum concentrations of 25–50 ng/ml appear to be adequate for efficacy (Lundmark and Reis, 2000; Mauri *et al.*, 2003). In contrast, Hong Ng *et al.* (2006) have indicated no visible relationship between plasma sertraline levels and its clinical efficacy. Nonetheless, it remains unclear whether there is a link between plasma sertraline levels and the clinical efficacy of sertraline.

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