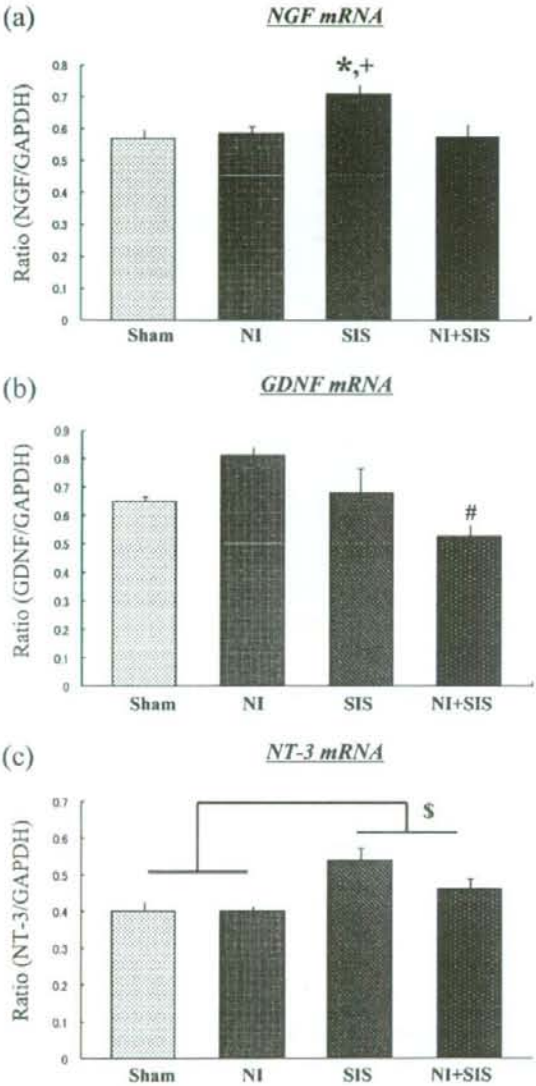

Fig. 2. Expression of (**a**) NGF, (**b**) GDNF, and (**c**) NT-3 mRNA in the hippocampus of adult rats subjected to sham treatment (Sham), neonatal isolation alone (NI), single immobilization stress alone (SIS), and neonatal isolation followed by a single immobilization stress (NI + SIS). Results are expressed as the ratio of the concentration of the target neurotrophin to that of GAPDH (target neurotrophin/GAPDH) percentage of Sham levels. The mean ± SEM (n = 6–8) is shown. $^{*}P < 0.05$ compared with sham, $^{\#}P < 0.05$ compared with NI, $^{+}P < 0.05$ compared with NI + SIS, $^{\$}P < 0.05$ compared with Sham and NI (two-way ANOVA followed by Scheffe's test).

In both juvenile and adult rats, two-way ANOVA showed no significant effect of NI on the level of NT-3 mRNA [juvenile: $F(1, 23) = 1.084$, $P = 0.309$; adult: $F(1, 23) = 2.539$, $P = 0.125$], but did show a significant effect of SIS [juvenile: $F(1, 23) = 13.285$, $P = 0.001$; adult: $F(1, 23) = 15.136$, $P = 0.001$], but no significant interaction between NI and SIS [juvenile: $F(1, 23) = 0.611$, $P = 0.443$; adult: $F(1, 23) = 2.319$, $P = 0.141$] (Figs. 1c and 2c). Posthoc analysis revealed that the level of NT3 mRNA in rats subjected to SIS was significantly higher ($P < 0.05$) than that in juvenile and adult rats not subjected to SIS.

DISCUSSION

The results of the present study demonstrate that repeated episodes of neonatal isolation do not affect the basal levels of NGF, GDNF, or NT-3 mRNA in the hippocampus of juvenile or adult rats not subsequently subjected to immobilization stress. However, we did observe a significant effect of NI on the basal level of GDNF mRNA in juvenile rats. The neonatal isolation paradigm used in this study has been reported to alter plasma corticosterone responses to restraint stress in juveniles and adults (Erabi et al., 2007; McCormick et al., 2002). This paradigm has also been reported to alter the levels of insulin-like growth factor-I (IGF-1) receptor and IGF binding protein-2 in the hippocampus in response to adulthood restraint stress (Erabi et al., 2007). Thus, it is likely that this neonatal isolation paradigm can affect the expression of neurotrophins in response to immobilization stress later in life.

Two studies conducted by Crelli et al. (1998, 2000) examined the influence of early adverse experiences on the expression of NGF in the rat hippocampus. The former study showed that a single brief episode of maternal separation (45 min) on PN day 3 significantly increased the level of NGF mRNA in the hippocampus of 3-day-old rats (Cirulli et al., 1998). Similarly, the latter study indicated that a single 1-h episode of maternal separation on PN day 9 or a single 3-h episode of maternal separation on PN day 16 significantly increased NGF mRNA in the DG (Cirulli et al., 2000). However, we found no significant influence of neonatal isolation on expression of NFG mRNA in the hippocampus of 28- or 90-day-old rats, and it may be that the effects of early adverse experience may no longer manifest at PN day 28. Further studies are necessary to determine whether there is a specific period of time during which neonatal isolation can affect the levels of NGF mRNA in the hippocampus. In contrast to studies using NGF (Cirulli et al., 1998, 2000), there has been no previous study examining the influence of early adversity on the basal levels of GDNF and NT-3 mRNA in the adult rat hippocampus. We have shown here that the level of neither GDNF nor NT-3 mRNA is altered in the hippocampus of 28- or 90-day-old rats that had been subjected to repeated episodes of neonatal isolation. In contrast to what has been reported for BDNF (Roceri et al., 2002, 2004), we did not find any change in the basal level of

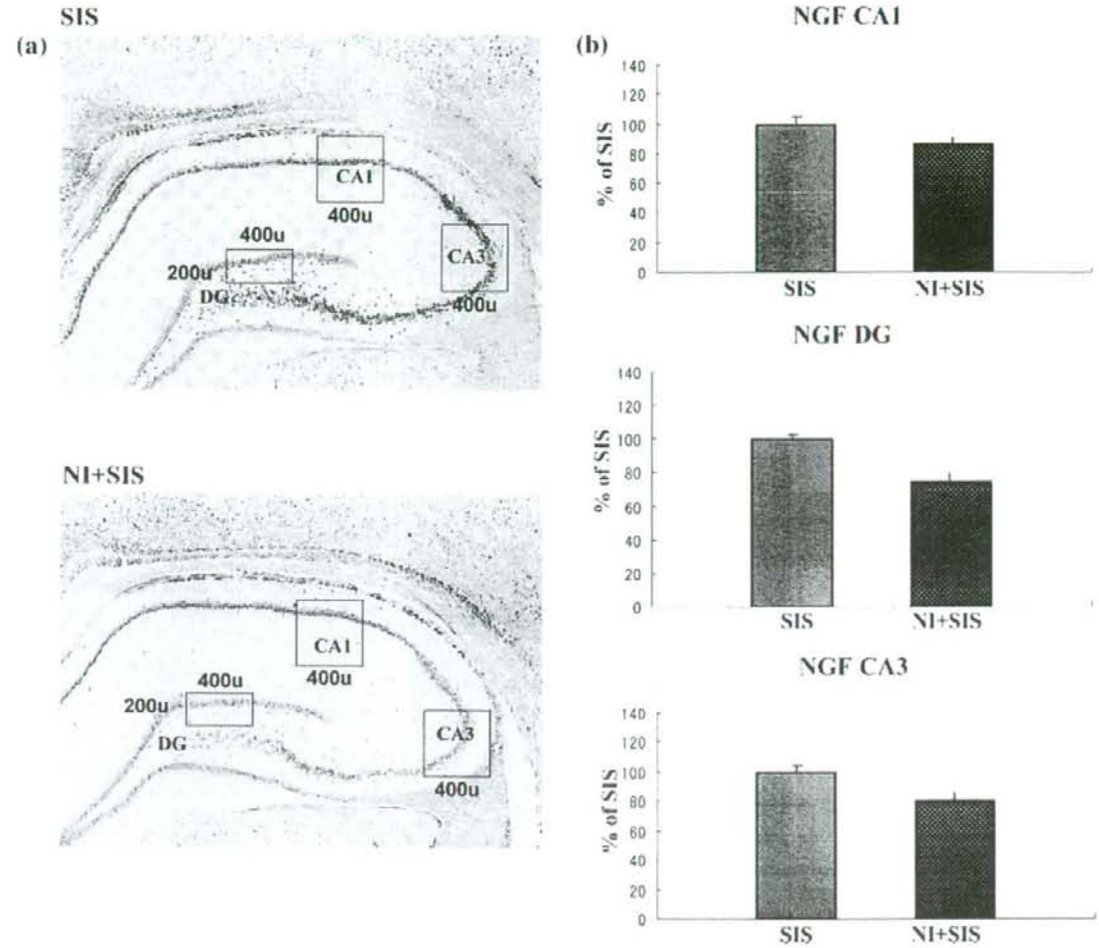

Fig. 3. Analysis of NGF mRNA expression by *in situ* hybridization in the adult rat hippocampus. (**a**) NGF mRNA expression in the rat hippocampus. SIS, single immobilization stress, NI + SIS, neonatal isolation followed by a single immobilization stress. (**b**) Mean density of NGF mRNA in the CA1 and CA3 pyramidal cell layers, and the dentate gyrus granular cell layer in the hippocampus of adult rats subjected to single immobilization stress (SIS) and neonatal isolation followed by a single immobilization stress (NI + SIS). Results are expressed as the percentage of SIS levels. The mean ± SEM (SIS: n = 7; NI + SIS: n = 6) is shown. $^{*}P < 0.05$ compared with SIS (Mann-Whitney U test). CA, cornu anmonis; DG, dentate gyrus.

NGF, GDNF, or NT-3 mRNA in the hippocampus of juvenile or adult rats that had been subjected solely to repeated episodes of neonatal isolation.

In this study, we also observed that exposure of rats to neonatal isolation abrogated the increase in the level of NGF mRNA that subsequently occurs in the same rats exposed to a single episode of immobilization as juveniles or adults. Furthermore, the exposure of rats to neonatal isolation decreased the level of GDNF mRNA in response to a single immobilization in juvenile rats. Recently, maternal separation (from Day PN 2 to PN day 14) has been shown to affect the level of NGF and NT-3 in the hippocampus of rats in response to single swim stress on PN day 35 and PN day 60 (Faure et al., 2006). Faure et al., (2006) demonstrated that adolescent and adult rats subjected to the swim stress expressed significantly higher levels of NGF and NT-3 in the hippocampus if they had been previously subjected to repeated episodes of maternal separation. Unfortunately, since they did not evaluate the influence of swim stress on the levels of NGF and NT-3 in the hippocampus of adult rats that had not been subjected to maternal separation as neonates, it is difficult to determine the effect of repeated episodes of maternal separation on the response of NGF and NT-3 to swim stress during adulthood.

Whereas subjection of adult rats to a single 8-h episode of immobilization stress leads to a significant

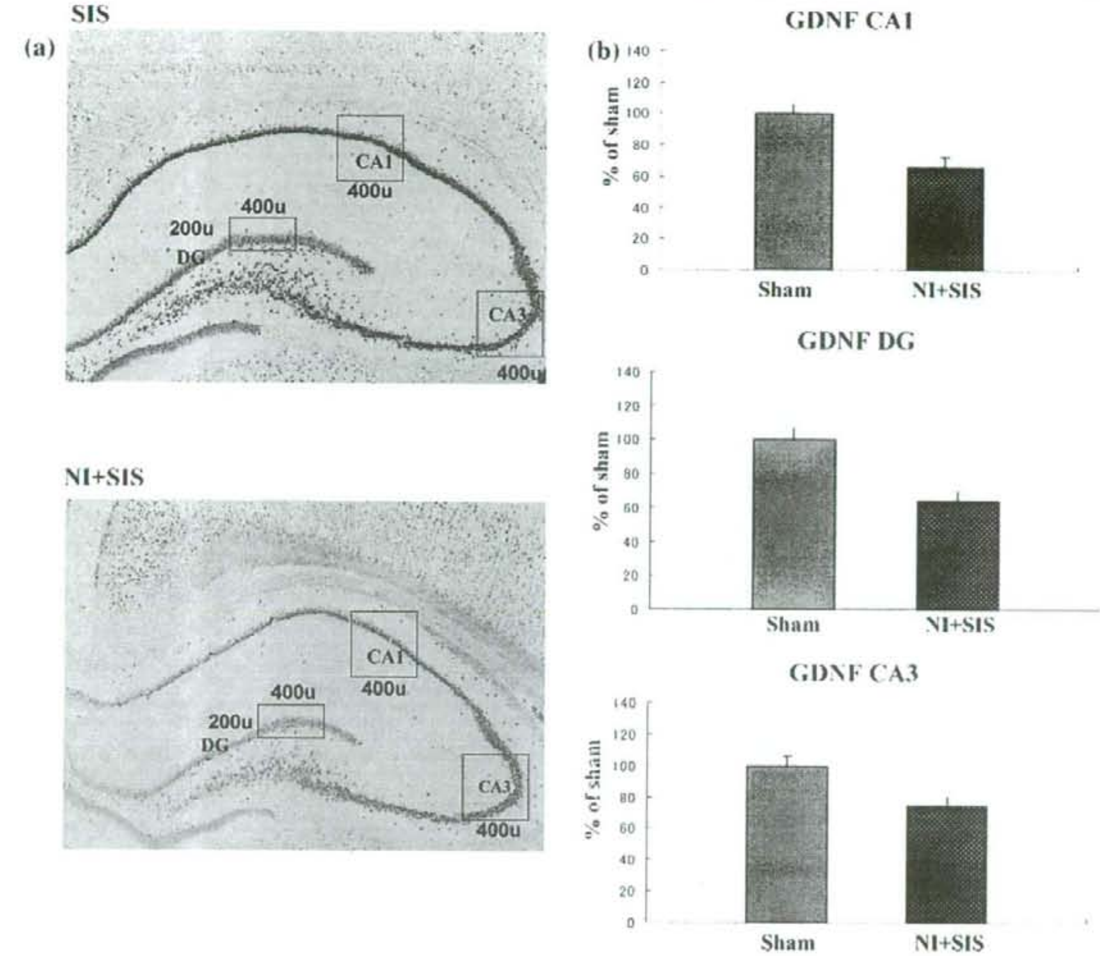

Fig. 4. Analysis of GDNF mRNA expression by in situ hybridization in the adult rat hippocampus. (**a**) GDNF mRNA expression in the rat hippocampus. NI, neonatal isolation; NI + SIS, neonatal isolation followed by a single immobilization stress. (**b**) Mean density of GDNF mRNA in the CA1 and CA3 pyramidal cell layers, and the dentate gyrus granular cell layer in the hippocampus of adult rats subjected to neonatal isolation (NI) and neonatal isolation followed by a single immobilization stress (NI + SIS). Results are expressed as the percentage of the SIS levels. The mean ± SEM (NI: $n = 7$; NI + SIS: $n = 6$) is shown. $^*P < 0.05$ compared with NI (Mann-Whitney *U* test). CA, cornu anmonis; DG, dentate gyrus.

reduction in the level of NGF in the hippocampus (Ueyama et al., 1997), traumatic brain injury (Grundy et al., 2001), subchronic cold stress (Foreman et al., 1993), immobilization stress (Smith and Clazza, 1996; Smith et al., 1995), and glucocorticoids (Mocchetti et al., 1996; Sun et al., 1993) significantly increase the expression of NGF in the rat hippocampus. These data indicating that stress-induced activation of the hypothalamo-pituitary-adrenal (HPA) axis upregulates the expression of NGF in the hippocampus, are consistent with the present findings. On the basis of the functional properties of NGF (Semkova and Kriegsten, 1999; Sofroniew et al., 2001; Tabakman et al., 2005), the induction of NGF in response to stress may be associated with neuroprotective responses. As such, the decreased response of NGF induction in both juvenile and adult rats and the decreased level of GDNF in juvenile, but not adult, rats in response to immobilization stress in the adult rats subjected to neonatal isolation may be, at least in part, involved in the stress susceptibility in later life due to neonatal isolation.

It is unclear how neonatal isolation attenuates the increased expression of NGF and GDNF in response to adulthood immobilization stress. Since activation of the HPA axis increases NGF expression in the rat brain (Mocchetti et al., 1996; Sun et al., 1993), it is hypothesized that the decreased response of the HPA axis to adulthood immobilization may account for the decreased induction of NGF in the rats subjected to neonatal isolation. However, we have recently reported that the corticosterone response following a single 2-h restraint stress in adult rats previously subjected to neonatal isolation was significantly enhanced compared with the effect in adult rats not previously subjected to neonatal isolation (Erabi et al., 2007). On the basis of our recent finding, it is unlikely that the HPA axis is involved in attenuation of the induction of NGF in adult rats that had been previously subjected to neonatal isolation.

Early adverse experiences have been suggested to alter epigenetic programming through DNA methylation and histone acetylation (Weaver et al., 2004). Weaver et al. (2004) demonstrated that low levels of maternal care markedly induced cytosine methylation in the promoter of the glucocorticoid receptor (GR). This increased methylation inhibited the stimulation of GR mRNA expression in response to restraint stress in adult rats, due to inhibition of the binding of NGF1A to the GR promoter. In analogy, the induction of cytosine methylation in transcription-factor binding regions of the promoters of NGF or GDNF genes following neonatal isolation might lead to a reduction of the induction of NGF and GDNF in response to immobilization stress in adult rats. Further studies examining the effect of neonatal isolation on cytosine methylation in the promoters of these genes are needed to elucidate the mechanism of the inhibition of gene induction.

In summary, repeated episodes of neonatal isolation inhibit the induction of NGF in the hippocampus of juvenile and adult rats, and decrease the levels of GDNF in the hippocampus of juvenile rats in response to a SIS, but have no significant effect on the basal expression of these mRNAs in the absence of immobilization stress. These findings suggest that the susceptibility to stress derived from experience of neonatal isolation might play a role in decreased neuroprotective support in response to subsequent exposure to stress later in file through the down regulation of NGF induction and decreased expression of GDNF.

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Regular Article

'Insistence on recovery' as a positive prognostic factor in Japanese stroke patients

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Aim: The present study used two-step analyses to examine the effect of acceptance of disability or 'insistence on recovery' in Japanese stroke patients: first on their functional improvement and second, on their psychological symptoms.

Methods: Disability was assessed using functional independence measurements (FIM), examining the stage of acceptance of disability by observation using Fink's theory (from shock to defensive retreat, acknowledgement, and acceptance/change stage), and estimation of insistence on recovery (on a scale of 1–4) by observation. The differences over time and the effects on the improvement in their FIM were then assessed. Depression was measured using the Zung Self-rating Depression Scale (SDS); apathy was measured using the Apathy Scale (AS), and the correlation with the acceptance stage or insistence on recovery was analyzed.

Results: The acceptance stage and functional improvement progressed significantly, but insistence on recovery did not change significantly during hospitalization. Multiple regression indicated that the insistence on recovery score (but not the acceptance stage) was a good predictor of the degree of improvement in FIM (FIM gain per week) in the elderly group. Post-hoc testing showed that the SDS or AS score decreased from the first stage to the fourth stage (but increased at the third stage) of acceptance; whereas for insistence on recovery score, the SDS and AS scores decreased as insistence on recovery score changed from 1 to 3, and then increased as insistence on recovery score changed from 3 to 4.

Conclusions: The appropriate level of insistence on recovery reduced depression and apathy, resulting in enhanced improvement of disability after a stroke in elderly stroke patients.

Key words: acceptance of disability, elderly, functional independence measurement, insistence on recovery, stroke.

STROKE HAS BEEN described as a condition with a unique epidemiological profile, with high incidence and mortality rates, and in which a large proportion of survivors have significant but varying degrees of residual disability.[1,2] There are two types of improvement that occur after a stroke: neurological improvement and improvement in functional abilities or performance.[1] Neurological recovery depends upon the mechanism of the stroke and the location of the lesion. By contrast, the improvement of functional abilities, such as activities of daily living (ADL), depends upon the environment in which the stroke patient is placed and how much training and motivation there is for the patient to learn to become independent again in terms of self-care and mobility. The ability to perform ADL can improve through

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acceptance and training in the presence or absence of natural neurological recovery.[1–4] Stroke rehabilitation must therefore restore optimal physical, psychological, social, and vocational function to enable the patient to become a productive participant in the community.

Emotional responses to stroke have traditionally been thought to follow a natural course of evolution: an initial state of significant distress or depression that, over time and as a result of some active process of 'working through', resolves to a condition of acceptance and relative emotional harmony.[5,6] This process was thought to be a mourning process or acceptance of disability. Disabled stroke patients who refuse to accept their impairment, eventually realize they are sick but continue to think that they will soon get well.[5,6] They manifested this attitude through direct verbal report or inferred from behavior continuously and repeatedly (this attitude was similar to whining, and we termed this emotional status as 'insistence on recovery'). Insistence on recovery is thought to be a sign of denial and thus is regarded as an irrational belief. Denial is generally found in psychiatric illness, but it is also found in the mourning process among disabled stroke patients.[6] It was traditionally thought that patients with insistence on recovery, whose only goal is recovery, can be motivated to do any work perceived as aiding recovery; but are not motivated to learn to function as a disabled person, and so fail to gain the maximum benefit from rehabilitation services.[5–8] Thus insistence on recovery is considered a maladaptive status and is thought to be a target for psychotherapeutic intervention.[5–8] We have, however, experienced many stroke disabled patients who say repeatedly that they will recover someday, and who participate in rehabilitation and gain independence in ADL in the rehabilitation service. The question arises whether insistence on recovery is an irrational or rational belief. Although there is much popular and professional literature attesting to the veracity of stages of acceptance of disability, the empirical data do not support such a contention.[5] Moreover, insistence on recovery is regarded as counterproductive to maximizing functional abilities and enhancing quality of life,[5–8] and no previous paper has examined the nature of 'insistence on recovery'.

The aim of the present study was to evaluate the effect of the stage of acceptance of disability or insistence on recovery on functional independence in stroke patients. Aging is associated with a high incidence of physical impairment, functional disability and depression.[9,10] Therefore, we divided stroke inpatients into two age groups: middle-aged and elderly. Moreover, depression and apathy are common neuropsychiatric consequences of a stroke, and can be examined using self-reported tests: the Zung Self-rating Depression Scale (SDS) and the Apathy Scale (AS). To clarify the psychopathological aspects of insistence on recovery after a stroke, we also examined the correlation between insistence on recovery score and the SDS or AS score.

METHODS

Study design

This study consisted of two parts: first, the changes in 'acceptance of disability' and 'insistence on recovery' over time were estimated, and we examined the effect of these factors on functional recovery after a stroke. Second, we examined the psychopathological symptoms (depression and apathy) using self-reporting scales, and estimated the association between depression and 'acceptance of disability' or 'insistence on recovery' after a stroke.

Patients

The approval of institutional ethics committees was obtained for this prospective study. Informed consent for functional or psychological measurements, including acceptance of disability, was obtained on admission from all patients or from those authorized to give consent on their behalf. The subjects for the first study were 231 patients with hemorrhagic and occlusive stroke without subarachnoid hemorrhage, diagnosed on computed tomography (CT) and/or magnetic resonance imaging (MRI), who were admitted to the Nishi-Hiroshima Rehabilitation Hospital <3 months after suffering a stroke (range, 10–109 days; mean, 44 ± 21 days), and who were hospitalized for more than 1 month. The patients were divided into two age groups at admission: the middle-aged group (95 patients, age 40–65 years); and the elderly group (136 patients, aged ≥ 66 years).

For the second study the subjects, who completed the SDS and AS scales, included 237 patients after excluding patients with (i) a history of major psychiatric illness, such as major depression, bipolar disorder, schizophrenia, or schizoaffective disorder; (ii) subarachnoid hemorrhage; (iii) emergency discharge 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-

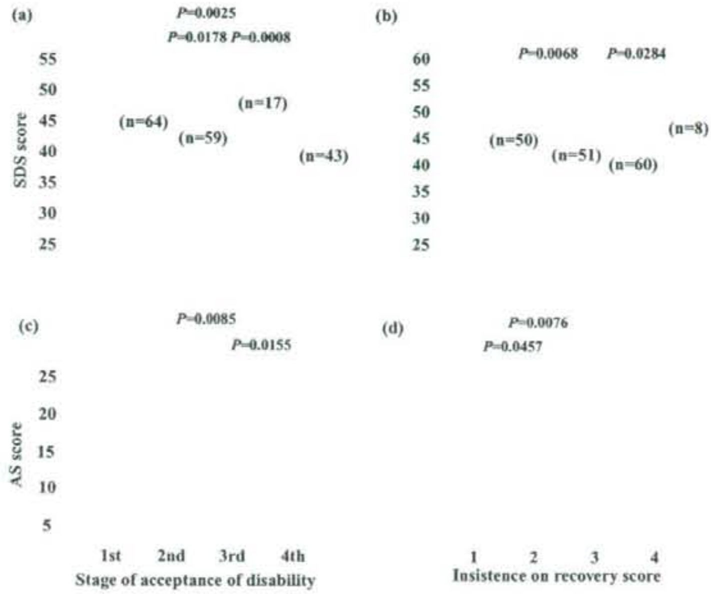

Figure 1. Differences in (a,b) Zung Self-rating Depression Scale (SDS) and (c,d) Apathy Scale (AS) score according to (a,c) acceptance stage (first, second, third, and fourth) and (b,d) insistence on recovery score (1–4). The data points are given as mean and 95% confidence interval. The Fisher protected 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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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.

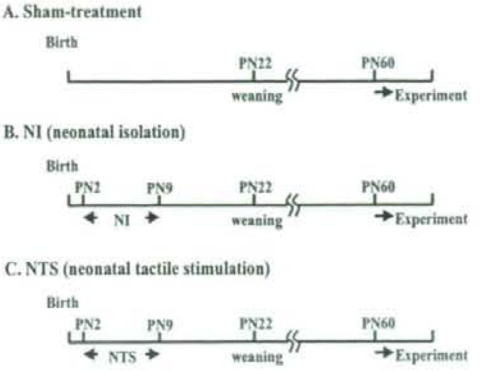

Fig. 1. Animal treatment paradigms. Prior to birth, litters from each dam were randomly assigned to (A) sham, (B) NI, and (C) NTS groups depending on neonatal treatments. All litters were weaned on postnatal (PN) day 22. After weaning, male and female rats in all groups were housed in same-sex, same-treatment 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	$2549.2 \pm 150.2^{*}$
Female	$2054.4 \pm 94.8^{\#}$	$1602.3 \pm 58.2^{\#}$	$2440.8 \pm 134.4^{*,\#}$
(b) Rearing			
Male	16.9 ± 2.1	$12.0 \pm 1.9^{\$}$	$27.8 \pm 2.7^{\$,\&}$
Female	15.8 ± 0.8	$10.2 \pm 0.9^{\$}$	$24.6 \pm 1.2^{\$,\&}$

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

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.

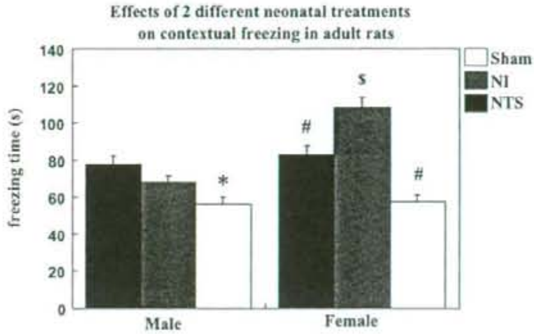

Fig. 2. Effects of two different neonatal treatments on contextual freezing time (s) in adult rats during a 180-s interval. Each group consisted of male (sham: $n=14$, NI: $n=14$, NTS: $n=16$) and female (sham: $n=15$, NI: $n=16$, NTS: $n=15$) rats. The results were expressed as the mean ± S.E.M. (*) 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].