Attenuated Anterior Cingulate Activation During a Verbal Fluency Task in Elderly Patients With a History of Multiple-Episode Depression

Hiroshi Takami, M.D., Yasumasa Okamoto, M.D., Ph.D., Hidehisa Yamashita, M.D., Ph.D., Go Okada, M.D., Ph.D., Shigeto Yamawaki, M.D., Ph.D.

Objective: *Previous studies have suggested that, in elderly patients, prior depression plays a role in the recurrence of depression. The aim of this study was to investigate cerebral brain function in recovered depressed elderly and investigate the relationship between this brain function and the number of depressive episodes.* **Methods:** *Twenty elderly depressive patients in recovery and 10 healthy volunteers were included in this study. The depressive patients were divided into those who had experienced a single depressive episode and those who had experienced multiple episodes. Functional magnetic resonance imaging was performed in each participant during a verbal fluency task. The data were analyzed using statistical parametric mapping.* **Results:** *Activation in the anterior cingulate cortex was significantly attenuated in patients who had experienced multiple depressive episodes, compared with the other two groups. There were no significant differences in areas of activation between patients with a single depressive episode and healthy volunteers.* **Conclusions:** *These findings suggest that attenuated activation in the anterior cingulate cortex may be associated with multiple episodes of depression in the elderly and with the vulnerability to cycling or recurrence.* (Am J Geriatr Psychiatry 2007; 15:594–603)

Key Words: Elderly depression, recurrence, cycling, anterior cingulate cortex, functional magnetic resonance imaging

In elderly patients with a history of depression, the risk of relapse is higher than in younger patients.[1–4] Evidence from several studies suggests that elderly patients with a history of multiple episodes of depression are at high risk of recurrence,[5,6] and that prior depression appears to be

Received August 6, 2006; revised September 22, 2006; accepted November 6, 2006. From the Department of Psychiatry and Neurosciences, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University (HT, YO, HY, SY) and Mihara Hospital (GO), Hiroshima, Japan. Send correspondence and reprint requests to Shigeto Yamawaki, MD, PhD, Department of Psychiatry and Neurosciences, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. e-mail: yamawaki@hiroshima-u.ac.jp.

an important risk factor for depression in the elderly.[7]

Treatment response and neuropsychological function also appear to be impaired in elderly patients who have experienced multiple depressive episodes compared with those who have experienced a single episode of depression.[8] Furthermore, multiple previous depressive episodes have been linked with neuropsychological impairment in the euthymic phase of depression.[9] These findings suggest that the presence of prior depressive episodes in elderly patients might affect their neuropsychological function and treatment response.

Previous functional neuroimaging studies in the depressed elderly[10–17] have suggested global and regional decreases in cerebral perfusion and glucose metabolism, particularly in the prefrontal and anterior cingulate cortex. However, results regarding the presence of this hypoperfusion and hypometabolism after remission and recovery from depression in the depressed elderly are inconsistent.

Although clinically the presence of a prior depressive episode appears to play a role in relapse and recurrence in elderly depression, the pathophysiological mechanisms involved are unclear. We hypothesized that there may be differences in the cerebral brain function of the depressed elderly that are related to the number of prior depressive episodes. However, to our knowledge, no previous functional neuroimaging study has been conducted to specifically examine this relationship. The aim of this study was to investigate the effect of the number of prior depressive episodes on cerebral brain function in recovered depressed elderly. Therefore, using functional magnetic resonance imaging (MRI) during a verbal fluency task which activates the prefrontal and anterior cingulate cortex,[18] we compared brain activity among patients with a single depressive episode, those with multiple episodes and healthy comparison subjects.

METHODS

Subjects

The study included 20 elderly patients in recovery from depression and 10 healthy volunteers. All subjects were Japanese, aged over 50 years and right-handed (according to the Edinburgh Handedness Inventory).[19] All depressed patients were outpatients at Hiroshima University Medical Hospital. They were received clinical interviews in a systematic manner by senior psychiatrists and diagnosed as having experienced one or more major depressive episodes according to *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM–IV)* criteria.[20] Exclusion criteria included a history of psychosis, mania, or other axis I disorder; history of neurological illness; and serious or unstable current medical condition. All of them had no psychiatric comorbidity and no family history of major depressive disorder in the first and the second degree of relationship. The age at onset of the first depressive episode in these 20 patients was over 50 years. They were in recovery and were being treated with average dosage of antidepressants for maintenance therapy at the time of this study. All antidepressants dosages were converted to an equivalent imipramine dosage according to Inagaki's report.[21] Recovery was defined as maintaining a score of no more than seven on the 17-item Hamilton Rating Scale for Depression[22] for over 3 months. Patients were divided into two groups according to the number of prior depressive episodes: a single-episode group (group 1: 10 subjects with a single depressive episode) and a multiple-episode group (group 2: 10 subjects with multiple depressive episodes). Of the patients in group 2, eight had experienced two depressive episodes and two had experienced three episodes. The last episode-free period was investigated for all patients and the score on the Global Assessment of Functioning scale was ascertained at the time of this study. The comparison group included 10 healthy volunteers comparable in age and education with the patients recruited from the local community. All of them had no current or lifetime psychiatric illness. No study subjects suffered from dementia as assessed by the Mini-Mental State Examination.[23] Detailed patient characteristics are outlined in Table 1.

The study was conducted under a protocol approved by the Ethics Committee of Hiroshima University School of Medicine. All subjects submitted informed written consent prior to their participation.

Experimental Paradigm

We used a periodic design involving the presentation of a baseline condition for 30 seconds followed by

TABLE 1. Demographic Details of the Three Groups

	Comparison	Single-Episode Group	Multiple-Episode Group	Degrees of Freedom	p Value
Number	10	10	10		
Sex (M/F)[a]	4/6	5/5	3/7	2	0.659
Age[b]	67.6 ± 9.7	65.9 ± 7.8	62.5 ± 9.1	2, 27	0.437
Year of education[b]	11.8 ± 2.6	12.1 ± 3.7	12.9 ± 2.1	2, 27	0.680
Score on MMSE[b]	29.0 ± 1.0	29.0 ± 1.0	28.8 ± 1.5	2, 27	0.899
Age at onset of first episode[c]		62.8 ± 7.7	57.0 ± 7.2	18	0.099
Months after last episode[c]		24.8 ± 18.9	23.9 ± 24.1	18	0.927
Score on HRSD[c]		3.2 ± 2.3	3.1 ± 2.2	18	0.922
Score on GAF scale[c]		81.0 ± 9.4	81.0 ± 8.8	18	0.999
Converted dosage of antidepressants (mg)[c]		49.0 ± 49.6	59.5 ± 54.2	18	0.657
Classes of antidepressants					
SSRI or SNRI/TCA or others/none)		3	4		
TCA or others		4	4		
None		3	2		
Adjuvant psychotropic drugs					
BDZs		7	7		
Anticonvulsants		0	0		
Antipsychotics		0	0		

Notes: Results are means ± SD. All antidepressants dosages were converted to an equivalent imipramine dosage. MMSE: Mini-Mental State Examination; HRSD: Hamilton Rating Scale for Depression; GAF: Global Assessment of Functioning; VFT: Verbal Fluency Task; SSRI: selective serotonin reuptake inhibitor; SNRI: serotonin norepinephrine reuptake inhibitor; TCA: tricyclic antidepressant; BDZ: benzodiazepine.

[a]Chi-square test.

[b]Analysis of variance.

[c]Unpaired *t*-test.

an activation condition for 30 seconds. This cycle was repeated three times over the course of three minutes. During the activation condition, subjects were cued by visual presentation of one of three letters (the Japanese phonetic characters that are pronounced "sa," "ta," and "te") and asked to generate a word beginning with that letter and to internally articulate the word but not to use the same word repeatedly. One of the three letters was presented every three seconds. During the baseline condition, subjects were cued by visual presentation of the word *yasumi* (which means "rest") every three seconds and asked to internally articulate that word. Before image acquisition, all subjects performed the following performance test outside of the scanner: using the same design as above with three different letters ("ka," "na," and "to" instead of "sa," "ta," and "te"). On this test, subjects were instructed to articulate the words externally, but not internally, and the number of words generated was recorded.

Image Acquisition

Functional MRI (fMRI) was performed using a Magnetom Symphony Maestro Class (Siemens, Tokyo) at Kajikawa Hospital. A time-course series of 64 image volumes was acquired with T2*-weighted, gradient echo, echo planar imaging (EPI) sequences. Each image volume consisted of 38 slices; the slice thickness was 4 mm with no gap, and covered the entire cerebral and cerebellar cortices. The interval between two successive acquisitions of the same image (TR) was 3,000 msec, the echo time (TE) was 48 msec, and the flip angle was 90°. The field of view (FOV) was 256 mm, and the matrix size was 64×64, giving voxel dimensions of 4×4×4 mm. Scan acquisition was synchronized to the onset of the trial. After functional scanning, structural scans were acquired using a T1-weighted gradient echo pulse sequence (TR = 2,050 msec; TE = 3.93 msec; flip angle = 15°; FOV = 256 mm; voxel dimensions of 1×1×1 mm), which facilitated localization.

Data Analysis

For statistical analysis, data were subjected to analysis of variance (ANOVA), chi-square test, and unpaired *t*-test for comparison of the demographic de-

tails of each group. Significance was defined as $p < 0.05$.

For image processing and statistical analyses we used the statistical parametric mapping (SPM99) software (Wellcome Department of Cognitive Neurology, London) implemented in Matlab (Mathworks Inc., Sherborn, MA). The first image volume was discarded because magnetization was unsteady, and the last three image volumes were discarded as they were taken during the posttask period. The remaining 60 image volumes were used for statistical analysis. Images were corrected for motion and realigned with the first scan of the session as a reference. T1 anatomical images were registered to the first functional image in each subject and aligned to a standard stereotaxic space, using Montreal Neurological Institute (MNI) T1 template in SPM99. The calculated nonlinear transformation was applied to all functional images for spatial normalization. Finally, the functional images were smoothed with a 10-mm full-width, half-maximum Gaussian filter.

Using group analysis according to a random effect model that allowed inference to the general population,[24] we identified brain regions that showed significant responses during word generation compared with word repetition. The group analysis consisted of two levels. In the first level, the signal time course of each subject was modeled with a delayed box-car function convolved with a hemodynamic response function in the context of a general linear model. One contrast image per subject was created by contrasting word generation with word repetition. In the second step, these images were entered into a one-sample *t*-test and then a two-sample *t*-test. The resulting foci were then characterized in terms of spatial extent (k) and peak height (u). The significance of each region was estimated using distribution approximations from the theory of Gaussian fields. This characterization was in terms of the probability that a region of the observed number of voxels (or greater) could have exceeded the determined threshold by chance (extent threshold), or that the peak height found (or higher) could have occurred by chance (height threshold) over the entire volume analyzed. Activations were reported if they exceeded $p < 0.001$ (uncorrected) on the single voxel level and $p < 0.05$ (corrected) on the cluster level. Significance on the cluster level was calculated considering peak activation and extent of the cluster.

The xyz coordinates provided by SPM, which are in the MNI brain space, were converted to xyz coordinates in Talairach and Tournoux's (TT) brain space[25] using the following formulas: $TT - x = MNI - x \times 0.88 - 0.8$; $TT - y = MNI - y \times 0.97 - 3.32$; $TT - z = MNI - y \times 0.05 + MNI - z \times 0.88 - 0.44$. Labels for brain activation foci were obtained in Talairach coordinates using the Talairach Daemon software, which provides accuracy similar to that of neuroanatomical experts.[26] The areas identified as labeled areas by this software were then confirmed by comparison with activation maps overlaid on MNI-normalized structural MRI images.

RESULTS

Clinical Background

As shown in Table 1, there were no statistically significant differences among the three groups in terms of gender, age, years of education, or score on Mini-Mental State Examination. There were no statistically significant differences between the two groups of depressive patients with respect to age at onset of first depressive episode, last episode-free period, score on Hamilton Rating Scale for Depression (HRSD), score on the Global Assessment of Functioning (GAF) scale, or dosage of antidepressants prescribed at the time of this study.

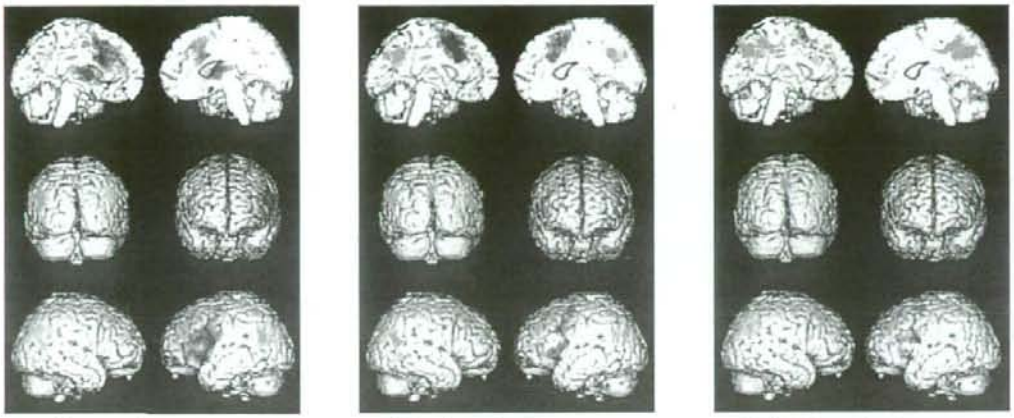
Behavioral Data

There was not a statistically significant between-group difference in the number of words generated in a verbal fluency task: single-episode group, 12.2 ± 2.5 words; multiple-episode group, 12.1 ± 3.2; comparison group, 11.9 ± 2.0 (ANOVA, $F=0.034$, $df=2,27$, $p=0.966$).

Neuroimaging Data

A one-sample *t*-test for each group revealed significantly increased activation in several brain regions involving the prefrontal cortex and anterior cingulate cortex, and significantly decreased activation in different several brain regions involving the parietal and temporal cortex (Figure 1, Table 2).

FIGURE 1. Statistical Parametric Maps of Brain Regions with Significant Activation and Deactivation During a Verbal Fluency Task in Each Three Groups



Comparison group | Single-episode group | Multiple-episode group

All activations were significant at a statistical threshold of $p < 0.001$ (uncorrected) on the single-voxel level and $p < 0.05$ (corrected) on the cluster level. Red scale represents increases and green scale decreases in activation. For exact coordinates, see Table 2. The images are projections of the significant points onto a normalized brain template.

Direct comparison by a two-sample *t*-test at each voxel of the brain activation among the three groups, showed that the comparison group had significantly greater activation than the multiple-episode group in the putamen, the left lateral globus pallidus, the anterior cingulate cortex and the right medial frontal cortex, and that the single-episode group had significantly greater activation than the multiple-episode group in the anterior cingulate cortex (Figure 2, Table 3). There were no significant differences in activation levels between the single-episode group and the comparison group at any brain region.

CONCLUSIONS

This is the first functional neuroimaging study undertaken to examine the relationship between number of previous depressive episodes in recovered depressed elderly and cerebral brain function. The major finding in this study was attenuated anterior cingulate activation in recovered depressed elderly patients with a previous history of multiple depressive episodes, compared with both those with a single episode and healthy volunteers. In contrast, there were no significant differences between patients with a single depressive episode and healthy volunteers.

To our knowledge, no study has reported a direct association between the number of prior depressive episodes and anterior cingulate function. However, two previous studies reported on the association between the number of depressive episodes and cognitive function. Driscoll et al.[8] reported that patients with late-onset, recurrent depression demonstrated more cognitive impairment on the Mattis Dementia Rating Scale compared to the late-onset, single-episode group. Also, Kessing[9] reported that the number of depressive episodes was correlated with cognitive decline on the Cambridge Cognitive Examination, the Global Deterioration Scale, and the like. Our major finding was attenuation of anterior cingulate cortex activation in depressed elderly with a history of multiple episodes of depression. Anterior cingulate cortex activation is related to the regulation of emotion and cognition,[27] and to the attentional demands of cognitive tasks such as the verbal fluency

TABLE 2. Areas With Significant Activation During a Verbal Fluency Task in Each Group

	Cluster Level		Voxel Level					
	Broadmann's Area	p Value	Number of Voxels in Cluster	p Value	T Score	X Coordinate	Y Coordinate	Z Coordinate
Comparison group[a]								
Left inferior frontal gyrus	47	0.000	20,051	0.009	20.21	−36	35	-4
Left middle frontal gyrus	46			0.009	20.06	−41	16	18
Left cingulate gyrus	24			0.012	19.05	−13	6	44
Right cerebellum		0.000	329	0.060	14.41	24	−63	−25
Right middle frontal gyrus	6	0.005	163	0.442	9.63	26	1	49
Right subgyral	6			0.989	6.39	19	1	53
Left fusiform gyrus	37	0.034	109	0.467	9.49	−41	−56	−10
Single-episode group[a]								
Left precuneus	7	0.002	246	0.034	15.15	−25	−63	33
Left superior frontal gyrus	6	0.000	8174	0.115	12.15	−6	10	51
Left medial frontal gyrus	32			0.126	11.95	−6	8	44
Left cingulate gyrus	24			0.154	11.49	−6	16	29
Right claustrum		0.000	732	0.416	9.27	33	8	−2
Right inferior frontal gyrus	47			0.528	8.69	31	22	−11
Multiple-episode group[a]								
Right claustrum		0.000	447	0.016	18.42	31	10	−3
Right inferior frontal gyrus	47			0.939	7.21	41	20	−6
Right insula	13			1.000	4.77	40	10	−5
Left claustrum		0.000	2,118	0.051	15.08	−31	10	0
Left insula	13			0.133	12.64	−40	3	19
Left precentral gyrus	6			0.169	12.06	−38	−3	28
Left superior frontal gyrus	6	0.000	1,227	0.097	13.40	−10	12	48
Left medial frontal gyrus	6			0.107	13.16	−1	−1	61
Left parahippocampal gyrus	36	0.035	103	0.412	9.95	−34	−29	−14
Left subgyral	20			0.990	6.48	−36	−13	−20
Right cingulate gyrus	24	0.001	197	0.634	8.82	19	−5	49
Right precentral gyrus	6			1.000	4.33	26	−15	48
Left middle occipital gyrus	19	0.001	205	0.657	8.72	−25	−81	15
Left lingual gyrus	17			0.966	6.92	−20	−83	3
Right cerebellum		0.000	470	0.748	8.30	27	−69	−28
Left fusiform gyrus	37	0.000	265	0.776	8.17	−36	−63	−7
Left cerebellum		0.002	186	0.791	8.09	−34	−60	−32

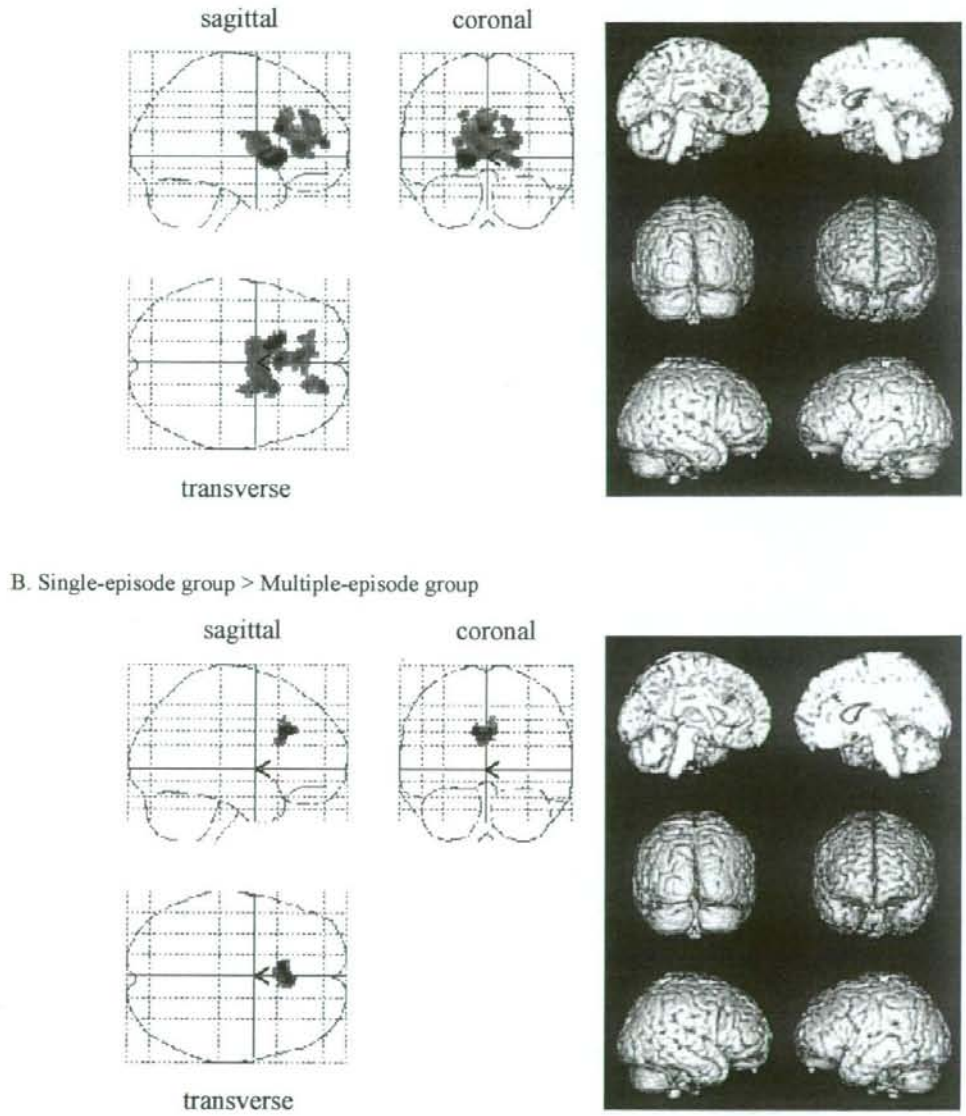
Notes: p values are corrected for spatial extent (cluster level p value) and peak height (voxel level p value) of the activation. All areas exceeding the corrected cluster level threshold of 0.05 are displayed. x, y, z coordinates: localization according to the standard Talairach coordinates (in mm).

[a]One-sample *t*-test (df=9).

task used in this study.[28] Furthermore, this region is thought to play a central role in normalization of the cerebral dysfunction that accompanies recovery from depression.[29] Our results suggest that there might be an important association between attenuated anterior cingulate activation and the number of depressive episode in depressed elderly.

Most functional neuroimaging studies[10–17] in elderly patients with depression, as well as in depression generally,[30–32] have consistently reported global and regional decreases in cerebral perfusion and glucose metabolism, particularly in the prefrontal and anterior cingulate cortex, while some studies have showed different results of increases in the left lateral prefrontal cortex, the bilateral orbital frontal cortex, etc.[33–35] However, previous studies on recovered depressed elderly have provided inconsistent results. Reduced regional cerebral blood flow has been demonstrated to be remaining[11–12] or diminished[17] after clinical recovery. Matsuo et al.[15] reported that, in recovered depressed elderly, activation in the prefrontal cortex during a verbal fluency task was significantly less than in controls. This inconsistency may be due to study designs limitations, where there were no references to the number of prior depressive episode or where studies included patients with varying numbers of depressive episodes. The results of our study showed that in recovery the dysfunction

FIGURE 2. Statistical Parametric Maps of Brain Regions with Significant Activation Differences During a Verbal Fluency Task Among the Three Groups



[A] The comparison group had greater activation than the multiple-episode group. [B] The single-episode group had greater activation than the multiple-episode group. All activations were significant at a statistical threshold of $p < 0.001$ (uncorrected) on the single-voxel level and $p < 0.05$ (corrected) on the cluster level. For exact coordinates, see Table 3. The images on the left of each figure are through-projections onto representations of standard stereotactic space (sagittal, side view; coronal, view from back; transverse, view from above). The images on the right are projections of the significant points onto a normalized brain template.

TABLE 3. Areas With Significant Activation Differences Among the Three Groups During a Verbal Fluency Task

	Cluster Level		Voxel Level					
	Broadmann's Area	p Value	Number of Voxels in Cluster	p Value	T Score	X Coordinate	Y Coordinate	Z Coordinate
Increased more in comparison group than in multiple-episode group [a]								
Left putamen		0.000	1,229	0.006	8.71	−18	16	1
Left lateral globus pallidus				0.016	7.96	−11	6	−3
Right putamen				0.446	5.59	17	10	−3
Left anterior cingulate gyrus	33	0.000	793	0.091	6.77	−3	14	20
Left anterior cingulate gyrus	24			0.382	5.73	−6	30	7
Right medial frontal gyrus	10	0.016	202	0.238	6.09	15	47	13
Right anterior cingulate gyrus	32			0.968	4.48	15	35	12
Increased more in single-episode group than in multiple-episode group [a]								
Left anterior cingulate gyrus	32	0.034	195	0.555	5.28	−10	18	29
Right anterior cingulate gyrus	32			0.741	4.96	1	22	27
Left anterior cingulate gyrus	24			0.971	4.35	−4	16	22

Notes: No areas were indicated for increased more in multiple-episode group than in single-episode group, increased more in single-episode group than in comparison group, increased more in comparison group than in single-episode group, increased more in multiple-episode group than in comparison group. p values are corrected for spatial extent (cluster level p value) and peak height (voxel level p value) of the activation. All areas exceeding the corrected cluster level threshold of 0.05 are displayed. x, y, z coordinates: localization according to the standard Talairach coordinates (in mm).

[a] Two-sample *t*-test (df=18).

in anterior cingulate cortex was revealed not in patients with a single depressive episode but in patients with multiple episodes. Therefore, our results provide new evidence for an association between cerebral brain function and the cycling of depressive episodes in recovered depressed elderly. Another possible explanation of these findings may be the vulnerability to recurrence. However, from the present study, we cannot conclude both possibilities.

In addition to the anterior cingulate cortex, attenuated activation was found in the basal ganglia and the medial frontal cortex in patients with multiple depressive episodes compared with healthy volunteers, although there was no attenuation in activation of these areas compared with single-episode depression. These areas are included in the front subcortical pathways which appear to play an important role in depression.[30,36–38] The attenuated activation in the anterior cingulate cortex, basal ganglia and the medial frontal cortex might be jointly associated with the dysfunction of these front subcortical pathways and with a risk of recurrence of depression in the elderly.

There was one contradictory finding in our study, in that we failed to find a difference in the number of words generated on the verbal fluency task between the three groups, even though there were significant differences in regional brain function. However, this may be explained by the higher sensitivity of functional neuroimaging over behavioral measures for the measurement of cognitive processing.[39] Another explanation of this finding may be a possibility that the patients with multiple depressive episodes were able to produce comparable numbers of words by activating more brain regions other than anterior cingulate cortex compared to two other groups (Table 2).

Our study has some limitations. First, we did not perform a structured interview (such as Structured Clinical Interview for *DSM–IV*) during the selection of subjects for participation in the study. Nevertheless, their diagnoses were made cautiously by means of semistructured clinical interview according to *DSM–IV* criteria with two senior psychiatrists, review of the patients' hospital records, and discussions with their clinical psychiatrist. Second, our findings may be limited by potential medication effects. However, there was no significant difference between the two groups of patients with respect to the dosage of antidepressants prescribed at the time of this study. And no established study has reported a negative influence of antidepressant medications on brain activity in a verbal fluency task, and numerous studies have demonstrated

that administration of antidepressants is not associated with a decrease in cerebral blood flow.[40,41] Third, the multiple-episode group was 5.8 years younger at their first episode than the single-episode group without statistical significance. Thus any differences between the two groups might be due to some function of the age of first episode or its duration, for example.

In conclusion, our results show that the attenuated anterior cingulate cortex activation may be associated with multiple depressive episodes in recovered depressed elderly, and with the vulnerability to cycling or recurrence. However, whether that attenuated activation results in or from the multiple episodes remains unclear. To answer this question, further longitudinal studies using the same subjects with elderly depression would be required.

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Depression or apathy and functional recovery after stroke

Seiji Hama[1]*, Hidehisa Yamashita[2], Masaya Shigenobu[1], Atsuko Watanabe[1], Keiko Hiramoto[1], Kaoru Kurisu[3], Shigeto Yamawaki[2] and Tamotsu Kitaoka[1]

[1]*Department of Rehabilitation, Nishi-Hiroshima Rehabilitation Hospital, Hiroshima, Japan*
[2]*Department of Psychiatry and Neuroscience, Graduate School of Biomedical Science Hiroshima University, Hiroshima, Japan*
[3]*Department of Neurosurgery, Graduate School of Biomedical Science Hiroshima University, Hiroshima, Japan*

SUMMARY

Objectives While depression and apathy are common after stroke, past studies have done little to examine the influence of these two symptoms on functional outcome respectively. This study was designed to examine the effect of depression or apathy on functional recovery after stroke in 237 Japanese stroke patients.

Methods We assessed the psychological status using self-rating scales [the Zung Self-Rating Depression Scale (SDS) for depression and the Apathy Scale (AS) for apathy] and an observer-rating scale [the Neuropsychiatric Inventory (NPI)]. We assessed physical disability using the Functional Independence Measurement (FIM). Post-hoc test and multiple regression analysis were used to determine the independent effects of post-stroke depression and apathy on functional outcome.

Results Depression was observed in 75 (31.6%) using SDS and 88 (40.2%) using NPI, and apathy in 95 (40.1%) using AS and 42 (19.2%) using NPI, respectively. Post-hoc test and multiple regression analysis indicated that the cognitive variable (Mini-Mental State Examination: MMSE score) and AS score, but not SDS score, correlated negatively with improvement in FIM.

Conclusions Apathy might be more frequently associated with functional abilities and likely interact with the recovery process as compared with depression after stroke.

KEY WORDS — apathy; depression; Zung Self-Rating Depression Scale; Neuropsychiatric Inventory; stroke; functional independent measurement

INTRODUCTION

Depression is a common neuropsychiatric consequence of stroke, affecting from 18–78% of patients during the acute and subacute phase of recovery (Singh *et al.*, 2000; Bhogal *et al.*, 2004). Several studies have shown that depression has a negative impact on recovery of activities of daily living (ADL) function in stroke patients (Singh *et al.*, 2000; Bhogal *et al.*, 2004). Apathy is also often observed after stroke and is defined as reduced motivation or lack of initiative and motivation (Starkstein *et al.*, 1993; Okada *et al.*, 1997; Yamagata *et al.*, 2004). The apathetic state after a stroke prevents patients from engaging in rehabilitation programs, resulting in delayed physical and social recovery.

The Zung Self-Rating Depression Scale (SDS) (Zung, 1965; Bhogal *et al.*, 2004; Kitamura *et al.*, 2004) and apathy scale (AS) (Starkstein *et al.*, 1993; Okada *et al.*, 1997; Yamagata *et al.*, 2004) are widely used, self-reporting questionnaire used to measure depression and apathy. However, the self-report measures (e.g. SDS, AS) resulted in a discrepancy with observer rating scale (e.g. DSM-III) diagnosis of depression, suggesting that either patients tend to minimize the severity of their mood disorders or examiners are sensitive to patients' behaviors (Gordon *et al.*, 1991; Bhogal *et al.*, 2004). The Neuropsychiatric Inventory (NPI) is an informant-based interview for evaluating behavioral changes following

*Correspondence to: Dr S. Hama, Nishi-Hiroshima Rehabilitation Hospital Miyake 265, Saeki-ku, Hiroshima 731-5143, Japan.
E-mail: wel@welnet.jp; shama@hiroshima-u.ac.jp

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the onset of illness, and it was shown to be the most useful for better assessing neuropsychiatric symptoms in stroke survivors (Cummings *et al.*, 1994; Angelelli *et al.*, 2004).

The aim of this study was to examine the extent to which post-stroke depression or apathy independently predicted poor recovery after stroke using both self-report measures (SDS and AS) and the observer rating scale (NPI).

METHODS

Patients

The approval of our institutional ethics committee was obtained for this prospective study. Informed consent was obtained from all patients. Subjects included in this study were selected from a consecutive series of 409 patients diagnosed with hemorrhagic or occlusive stroke using computed tomography (CT) who were admitted to the Nishi-Hiroshima Rehabilitation Hospital less than 3 months (7–90 days) after suffering their stroke. We excluded 35 patients with: (1) a history of major psychiatric illness, such as major depression, bipolar disorder, schizophrenia, or schizoaffective disorder ($n = 5$); (2) subarachnoid hemorrhage ($n = 24$); and (3) emergency discharge because of medical illness ($n = 6$). The apathy scale and SDS are patient-rated scales which 137 patients (36.6%) of the remaining 374 patients were unable to complete because of: (1) medications, medical illness, or physical disability affecting cognitive function or the ability to provide consent (speech impediment: $n = 123$, medical illness: $n = 2$); or (2) a physical disability which precluded cognitive testing ($n = 12$). The remaining 237 patients completed the SDS and AS and were included in the study. For 18 patients psychopathological information from caregivers could not be obtained; the remaining 219 patients had caregivers who completed the NPI scales.

The data were gathered from a subset sample of a previous study (Hama *et al.*, 2007) conducted as part of a larger program of research on depression and lesion location among stroke rehabilitation patients. Patients were not selected for on the basis of any of the study variables.

Treatment

This unit provides intensive multidisciplinary goal-oriented inpatient rehabilitation. The staff of medical doctors, nurses, care workers (CW), physical therapists (PT), occupational therapists (OT), speech therapists (ST), medical social counselors (MSC) and clinical psychotherapists (CP), assembled every 1–2 weeks in the conference room to devise individual care plans for the physical, psychological, and social support for each inpatient, and to review the patients' rehabilitation programs.

CT findings

CT scanning was performed on all patients on admission and follow-up scans were performed every 1–3 months after admission to measure the infarction/hemorrhage site and volume (cubic centimeters). The measurement was calculated according to the formula $0.5 \times A \times B \times C$; where A and B represent the largest perpendicular diameters through the hypodense area on CT scan, and C is the thickness (C; calculated by slide width $\times$ slice numbers) of the infarction area (Montaner *et al.*, 2001).

Functional measures

The functional independence measurement (FIM) 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 (Christiansen and Ottenbacher, 1998; Tsuji *et al.*, 2000). The maximum total FIM score is 126; the lower the score, the greater the disability. All patients were examined for disability using the FIM (version 3.0, translated into Japanese) within 1 week of admission and at 1–2 week intervals during hospitalisation.

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

Motor impairment in hemiplegic stroke patients was assessed using the Brunnstrom Recovery Scale (BRS), in which movement patterns are evaluated and motor function is rated according to stages of motor recovery (Brandstater, 1998). The BRS defines recovery only in broad categories that correlate with progressive functional recovery.

Cognitive functions of stroke patients were measured using the Mini-Mental State Examination (MMSE; score range 0–30).

Self-Rating Depression Scale (SDS)

We used the Japanese version of the SDS to examine the subjective severity of depression. SDS was performed 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 (Yamaguchi *et al.*, 1992).

Apathy Scale (AS)

To quantify the apathetic state, we used a Japanese version of the Apathy Scale developed by Starkstein *et al.* (Starkstein *et al.*, 1993; Okada *et al.*, 1997; Yamagata *et al.*, 2004). The AS was performed 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 against four grades (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 (apathy score <16 points) and an apathetic group (apathy score ≥16 points). In the previous study, Okada *et al.* (1997) performed a reliability test for the Japanese version of AS described as follows; a normal control subjects had a low AS score. For 50 stroke patients, the clinical judgment of the existence of apathy by two neurologists was compared with the AS score. The most reliable results were obtained at a cutoff score of 16 points. The sensitivity of AS was 81.3%, and the specificity was 85.3%.

Japanese version of the Neuropsychiatric Inventory (NPI)

The NPI is based on a structured interview with a caregiver familiar with the behavior of the patient (Cummings *et al.*, 1994; Hirono *et al.*, 1997). The NPI was performed within 1 month of admission. The interviewer confirms whether the patient has any psychiatric symptoms (delusion, hallucination, agitation, dysphoria, anxiety, euphoria, apathy, disinhibition, irritability and aberrant behavior) and determines the severity and frequency of each symptom according to defined criteria. Frequency is rated on a five-point scale from 0–4 and severity is rated on a four-point scale from 0–3: the larger the score, the higher the severity or frequency. The product of the severity score and the frequency score gives the score for each symptom (Cummings *et al.*, 1994; Hirono *et al.*, 1997). We used the subscales of depression and apathy for this study.

Statistical analyses

Different degree of psychological status (no depression and apathy, depression only, apathy only, both depression and apathy) were compared with a post-hoc Fisher protected least significant difference test (Fisher PLSD test).

Multiple regression analysis was used to estimate the independent effects of predictor variables (apathy score, SDS score, BRS, size of CT, MMSE, age, sex, FIM score on admission, period of hospitalisation) on improvement in FIM (FIM gain/week).

Values were considered to be significant at $p < 0.05$. The Stat View 5.0 (SAS Institute, Inc., Cary, NC) statistical package was used for all analyses.

RESULTS

Baseline structures and the frequency of PSD in all patients

We used the SDS score to assess affective PSD, and the AS score to assess apathetic PSD. Table 1 shows baseline data for all patients. SDS and AS scores over the cutoff limits were observed in 75 (31.6%) and 95 (40.1%) patients, respectively. The NPI scale demonstrated that depression was exhibited by 40.2%, and apathy by 19.2% of all patients, respectively.

Post-hoc study for examine the effect of psychopathology on the FIM score

Post-hoc test (Fisher PLSD test) showed a statistical difference between none and apathy only ($p = 0.0486$), and none and both apathy and depression ($p = 0.0365$) (Figure 1A). However, we did not find significant differences in FIM improvement between none and depression.

Post-hoc test of the data of observer-rating test (NPI) showed no statistical difference, however the same tendency was existed between none and both apathy and depression group (Figure 1B, $p = 0.0839$). Therefore, it was suggested that the severity of apathy (not depression) might be associated with improvement in FIM.

The effects of the apathy or SDS score on improvement in FIM after stroke

To identify predictors of improvement in FIM after stroke, we performed multiple regression analysis with sex, age, period of hospitalisation, FIM at admission, BRS (upper limb, finger, lower limb), Lesion size (CT), depression, and apathy as independent variables,

Table 1. Baseline characteristics on admission

		Baseline ($n = 237$)
Age (yrs)	Mean (SD)	65.1 (11.3)
Gender (%)	Female	81 (34.2%)
Duration of hospitalization (days)	Mean (SD)	149.3 (49.2)
Time interval between onset and admission (days)	Mean (SD)	40.6 (19.7)
Type of stroke (hemorrhage; infarction)		109; 128
Past history of stroke (%)		27 (11.4%)
SDS score	Mean (SD)	40.3 (8.7)
Depressive state (%)		75 (31.6%)
NPI: depression subscore[a]	Mean (SD)	1.3 (2.1)
Depressive state (%)		88 (40.2%)
Apathy score	Mean (SD)	14.1 (8.1)
Apathetic state (%)		95 (40.1%)
NPI: apathy subscore[a]	Mean (SD)	1.0 (2.5)
Apathetic state (%)		42 (19.2%)
Size of LDA in CT (cm^3)[b]	Mean (SD)	26.9 (52.6)
BRS upper limb	Mean (SD)	3.3 (1.6)
BRS finger	Mean (SD)	3.3 (1.7)
BRS lower limb	Mean (SD)	3.6 (1.5)
MMSE[c]	Mean (SD)	24.3 (5.4)
FIM total	Mean (SD)	77.1 (22.4)
FIM motor	Mean (SD)	50.6 (18.0)
FIM cognitive	Mean (SD)	26.5 (6.8)

Continuous values are mean (SD), and categorical values are number of patients (percentage).
BRS = Brunnstrom Recovery Scale; FIM = Functional Independence Measurement; MMSE = Mini-Mental State Examination; SDS = Zung Self-rating Depression Scale.
[a]Eighteen patients in whom NPI examination was not carried out is not included in this line.
[b]Twelve patients in whom CT examination was not carried out is not included in this line.
[c]Twelve patients in whom MMSE examination was not carried out is not included in this line.

with improvement in FIM as the dependent variable. The data of self-rating scale (SDS, AS) showed that the FIM score on admission, the period of hospitalisation, age, MMSE, and the AS score were correlated significantly with FIM gain/week, while SDS score was not (Table 2). The NPI data also showed the same trend (NPI apathy: 0.0555, NPI depression: 0.1202) (Table 3). Therefore, it was noteworthy that the apathy (but not the depression) correlated negatively with improvement in FIM after stroke.

DISCUSSION

This study was undertaken to evaluate the relationships between functional outcome and two main psychological symptoms (depression and apathy) during rehabilitation in patients hospitalised after having a stroke using both self-rating scales and an observer-rating scale. We found that the apathy was a predictor of poor functional recovery after stroke.

Depression is a complicating factor in stroke rehabilitation, with depressed patients demonstrating poorer recovery from stroke (Singh *et al.*, 2000; Bhogal *et al.*, 2004). Cully *et al.* (2005) reported that depressive symptoms were related to functional recovery across multiple domains. Robinson *et al.* (1984) and Starkstein *et al.* (1987) examined depression in stroke patients using the SDS, and their findings suggested a strong correlation between the severity of depression and the proximity of the lesion to the frontal pole. We recently examined the location of lesions in stroke patients using CT scans and reported that depressed affect (estimated using SDS) was associated with lesions of the left frontal lobe (Hama *et al.*, 2007). These observations suggest a link between depression and anterior left cerebral hemisphere lesions.

Apathy is also reported to have a negative impact on recovery of ADLs in stroke patients. Starkstein *et al.* (1993) demonstrated that apathetic patients were significantly older with more severe cognitive impairment and had significantly greater deficits in ADLs than non-apathetic patients. Piamarta *et al.* (2004) demonstrated significant correlations between the Barthel index and affective symptoms (depression, apathy and emotional lability). In this study, we examined the extent to which severity of depression and apathy predicted recovery after stroke and demonstrated that apathy was a stronger predictor of poor recovery after stroke than depression.

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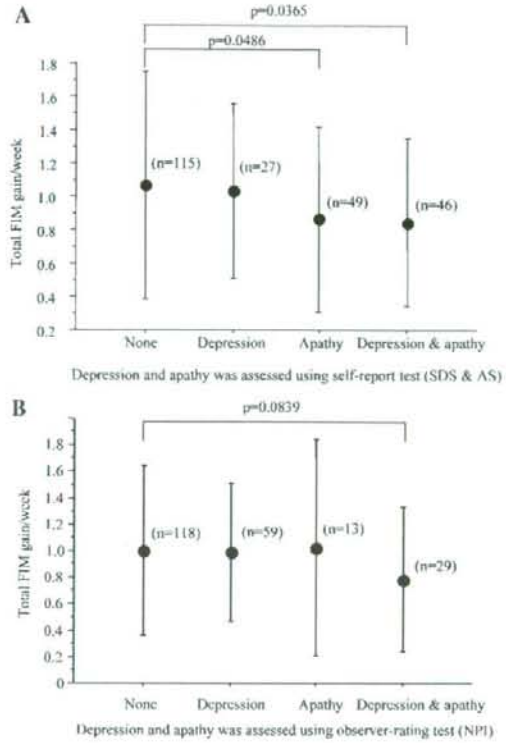

Figure 1. Differences in FIM gain/w between patients with no, depressive only, apathetic only, and both depressive and apathetic psychological symptoms were shown. The mid point, top and bottom of each vertical line represent the mean, upper, and lower 95% CI values, respectively. The Fisher PLSD test indicates that these parameters can distinguish between some of these psychological subgroup, with the p-values given between none and apathy only or both apathy and depression (A) and between none and both apathy and depression (B).

Starkstein *et al.* (1993) were the first to provide empirical data about apathy in stroke patients, reporting that apathy was associated with lesions involving the posterior limb of the internal capsule. Later, Okada *et al.* (1997) examined regional cerebral blood flow in Japanese stroke patients with apathy, demonstrating a link between symptoms of apathy after stroke and hypoactivity of the frontal and anterior temporal lobes. Yamagata *et al.* (2004) examined the relationship between apathy after subcortical stroke and neural orienting responses to novel events using an event-related evoked potential technique, and concluded that apathy after subcortical (including basal ganglia) stroke is linked to dysfunction of the frontal-subcortical system. Recently, Brodaty *et al.* (2005)

Table 2. Multiple regression analysis of FIM gain/w with predictable variables. Self-report test (SDS and AS)

	FIM gain/w	
	SC	*P*
Apathy score	−0.015	0.0079
SDS score	0.002	0.73
BRS upper limb	−0.029	0.6358
BRS finger	0.048	0.3531
BRS lower limb	0.063	0.1617
CT size	0.000	0.0064
MMSE	0.028	0.0014
Age	−0.009	0.0285
Sex	0.057	0.4745
Period of hospitalisation	−0.005	<0.0001
FIM score on admission	−0.021	<0.0001

Apathy and depression was estimated with self-report test (SDS and AS).
AS = Apathy Scale; BRS = Brunnstrom Recovery Scale; FIM = Functional Independence Measurement; MMSE = Mini-Mental State Examination; SDS = Zung Self-rating Depression Scale.

reported that apathy is associated with functional impairment and cognitive impairments, affecting concentration and information processing speed, and may be secondary to pathology affecting right-sided frontal-basal ganglia pathways. Glodzik-Sobanska *et al.* (2005) examined the association between apathy in stroke patients and proton magnetic resonance spectroscopy findings in their unaffected frontal lobes, and demonstrated a correlation between apathy and reduction in the N-acetylaspartate/creatine ratio in the

Table 3. Multiple regression analysis of FIM gain/w with predictable variables. Observer-rating test (NPI)

	FIM gain/w	
	SC	*P*
NPI: Apathy	−0.036	0.0555
NPI: Depression	−0.030	0.1202
BRS upper limb	0.013	0.837
BRS finger	0.015	0.7791
BRS lower limb	0.059	0.2194
CT size	0.000	0.0222
MMSE	0.028	0.0019
Age	−0.008	0.0589
Sex	0.047	0.5732
Period of hospitalisation	−0.004	<0.0001
FIM score on admission	−0.021	<0.0001

Apathy and depression was estimated with observer-rating test (NPI).
BRS = Brunnstrom Recovery Scale; FIM = Functional Independence Measurement; MMSE = Mini-Mental State Examination; NPI = Neuropsychiatric Inventory.

right frontal lobe. In our recent CT study (Hama *et al.*, 2007) we found that 'loss of interest' (apathy) was associated with bilateral lesions of the basal ganglia. These findings suggest that apathy after stroke is related to frontal-subcortical dysfunction involving the basal ganglia. We believe that this leads to reduced motivation and cognitive dysfunction (such as lack of attention), which in turn result in dependency in ADLs. As discussed previously, depression and apathy appear to have a different neuroanatomical basis, and can therefore be treated as separate phenomena in the prediction of recovery after stroke.

Several methodological limitations of this study should be acknowledged. Because patients with severe comprehension deficits were excluded from the study, the results may not be applicable to all stroke patients. We did not examine the time course of SDS and AS score, and the improvement or worsening of this psychopathology may have influenced our findings. Therefore, further study is needed to examine the effect of the psychopathological time course on the functional outcome. Additionally, because the sample size was modest the results require replication with a larger sample.

The findings from this study do not suggest that psychological symptoms cause functional deficits; rather they indicate that psychological symptoms, especially apathy, are frequently associated with functional abilities and likely interact with the recovery process. There are different underlying neuroanatomical and neurobiological mechanisms. In order to help patients gain independence and function, future studies should examine whether these differing associations to functional recovery correlates of depression and apathy, separately or together, may also be reflected in different patterns of treatment response.

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Prior Neonatal Isolation Reduces Induction of NGF mRNA and Decreases GDNF mRNA in the Hippocampus of Juvenile and Adult Rodents Subjected to Immobilization Stress

KI-ICHIRO KAWANO,[1] SHIGERU MORINOBU,[1]* TAKUYA SAWADA,[1] SEIICHI TSUJI,[1] KAORI ERABI,[1] MANABU FUCHIKAMI,[1] TOSHIRO KOZURU,[1] SHIGETO YAMAWAKI,[1] KAZUE HISAOKA,[2] AND MINORU TAKEBAYASHI[2]

[1]*Division of Frontier Medicine, Department of Psychiatry and Neurosciences, Graduate School of Medical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan*

[2]*Division of Clinical Research, Department of Psychiatry, National Hospital Organization, Kure Medical Center, 3-1 Aoyama, Kure, 737-0023, Japan*

KEY WORDS neonatal isolation; immobilization; NGF; GDNF; NT-3; hippocampus

ABSTRACT Numerous studies have demonstrated that early adverse experiences are associated with the development of susceptibility to stress later in life. Although it is known that early experience of adversity, such as neonatal isolation, maternal separation, and low maternal care, enhances the activity of the hypothalamo-pituitary-adrenal axis in rodents, the detailed mechanism underlying stress susceptibility induced by early adversity remains to be elucidated. Since neurotrophins have been shown to have a neuroprotective effect, we examined the influence of repeated neonatal isolation on expression of nerve growth factor (NGF), glia cell-derived neurotrophic factor (GDNF), and neurotrophin-3 mRNA in the hippocampus of juvenile and adult rats subsequently exposed immobilization stress, using real-time quantitative PCR and in situ hybridization. Neonatal isolation did not affect the basal hippocampal expression of these neurotrophin mRNAs in either juvenile or adult rats not subsequently exposed to immobilization. Similarly, there was a significant interaction between neonatal isolation and immobilization that affected the expression of NGF and GDNF mRNAs. Neonatal isolation attenuated the induction of NGF mRNA in both groups of rats and decreased GDNF mRNA in juvenile rats in response to immobilization. The decreased induction of NGF mRNA and reduced GDNF mRNA in response to immobilization was found in the CA3 pyramidal cell layer and dentate gyrus granular cell layer in the hippocampus of adult rats that had been subjected to neonatal isolation. These findings suggest that susceptibility to stress arising from prior neonatal isolation might be a result of decreased neuroprotective support through NGF and GDNF. **Synapse 62:259–267, 2008.**

INTRODUCTION

Numerous preclinical studies have revealed that exposure to either early adversity and/or long-term stress in adulthood can lead to alterations in hippocampal structure and function. For example, chronic adulthood stresses, such as forced immobilization or restraint, have been reported to induce a significant decrease in the dendritic length and the number of branch points in hippocampal CA3 pyramidal neurons (Vyas et al., 2002), and to atrophy of the apical dendrites of CA3 pyramidal cells in the rat hippocampus (Magarinos et al., 1995, 1996; Watanabe et al., 1992). Similarly, chronic parental separation induces a significant decrease in the length of the dendrites of hippocampal CA1 pyramidal neurons, and a significant reduction of granular cell spine density in the hippocampal dentate gyrus (DG) in the juvenile degus

*Correspondence to: Shigeru Morinobu, MD, PhD, Division of Frontier Medicine, Department of Psychiatry and Neurosciences, Graduate School of Medical Sciences, Hiroshima University, 1-2-3 Kasumi minami-ku, Hiroshima 724-8551, Japan. E-mail: smoriob@hiroshima-u.ac.jp

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(Poeggel et al., 2003). In addition, there is a significant reduction of mossy fiber density in the hippocampus of adult rats subjected to repeated episodes of maternal separation (Huot et al., 2002). Moreover, juvenile rats subjected to repeated episodes of maternal separation experienced a significant decrease in the volume and total number of BrdU-positive cells within the DG as well as a significant increase in the number of TUNEL-positive cells within this region of the brain (Lee et al., 2001). Mild prenatal stress has been shown to decrease synaptic density in the hippocampus of affected offspring (Hayashi et al., 1998). Taken together, these morphological findings suggest that various types of stress affect synaptic structure. However, the precise mechanism by which exposure to stress induces morphological changes in the hippocampus remains to be elucidated.

Neurotrophic factors and growth factors play a critical role in the differentiation and maintenance of neurons (Folli et al., 1996; Maisonpierre et al., 1990), suggesting that these factors may be involved in stress-induced synaptic remodeling. A significant reduction of brain-derived neurotrophic factor (BDNF) mRNA was found in the hippocampus of adult rats subjected to single and repeated episodes of maternal separation (MacQueen et al., 2003; Roceri et al., 2002). In contrast, the level of BDNF protein in adult rats subjected to repeated episodes of maternal separation was higher than that in adult rats subjected to neonatal brief handling despite a similar level of BDNF mRNA in these 2 groups (Greisen et al., 2005). Furthermore, the level of BDNF mRNA in the hippocampus of offspring of mothers that exhibited a high degree of maternal care was significantly higher than that in offspring of mothers that exhibited a low degree of maternal care (Liu et al., 2000). In contrast, a single episode of maternal separation was reported to increase the level of nerve-growth factor (NGF) mRNA in the developing rat hippocampus (Cirulli et al., 1998, 2000). Recently, we demonstrated that repeated episodes of neonatal isolation led to a significant decrease in the level of insulin-like growth factor (IGF) 1 receptor mRNA and protein in the hippocampus of the corresponding adult rats (Erabi et al., 2007). Although the influences of early adverse experiences on the expression of neurotrophic and growth factors in the hippocampus has been recognized, further studies focusing on other factors such as NGF, glia cell-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT-3), are required to elucidate the involvement of these factors in the morphological changes in response to early adversity.

In this report, we examined the effect of repeated episodes of neonatal isolation on the levels of NGF, GDNF, and NT-3 mRNA in the hippocampus of juvenile (28-day-old) and adult (90-day-old) rats. In addition, epidemiological studies of mental disorders have suggested that early adverse experiences increase the susceptibility to mental disorders in response to stress. Therefore, we also examined the levels of these neurotrophic factors in response to a single episode of immobilization stress (SIS) in juvenile and adult animals that had been previously exposed to repeated episodes of neonatal isolation.

MATERIALS AND METHODS

Animals

Pregnant female Sprague-Dawley rats were purchased from Charles River Japan (Yokohama, Japan). The rats were housed individually in a breeding colony at constant room temperature (23°C ± 2°C) and humidity (60%) with a 12 h/12 h light/dark cycle (lights on 8 AM to 8 PM). Food and water were provided ad libitum. The litters were culled to 12 pups on postnatal day 1 (PN day 1).

Neonatal isolation paradigm

The mothers and pups of the nonisolated group were left undisturbed until weaning. Neonatal isolation was conducted according to the method of Kehoe and Bronzino (1999). Pups were isolated from the dam, nest, and siblings and placed in individual round containers for 1 h per day on PN days 2–9. All litters were weaned on PN day 21, separated on the basis of sex, and maintained with ad libitum access to food and water. Only the male rats were subjected to the following experimental procedure. All animal procedures were conducted in accordance with the Guiding Principles on Animal Experimentations in Research Facilities for Laboratory Animal Science Hiroshima University and approved by Hiroshima University Animal Care Committee.

Single immobilization stress paradigm

On PN day 28 (juvenile) or 90 (adult), half of both the isolated and nonisolated rats were subjected to a single restraint stress (SRS) for 2 h. The immobilization stress was conducted as described previously (Morinobu et al., 2003; Suenaga et al., 2004). Briefly, rats were immobilized in clear polyethylene disposable cone bags (Asahikasei, Tokyo, Japan), sized so that rats were equally immobilized. Animals were sacrificed by decapitation after completion of the immobilization stress. On PN day 28 or 90, isolated and nonisolated rats (sham) were sacrificed by decapitation. The hippocampus was isolated, immediately frozen, stored at −70°C, and used for real-time quantitative PCR analysis. In this procedure, the lateral choroid plexus was carefully removed from the hippocampus. For in situ hybridization, brains were

TABLE I. Primer and probe sequences used for real time PCR

NGF	Forward primer	AGCCCACTGGACTAAACTTCAGC
	Reverse primer	GGGCACTGCGGGCTC
	TaqMan prove	TTCCCTTGACACAGCCCTCCGC
GDNF	Forward primer	ACTCCCTCGGCCACCG
	Reverse primer	ATAATCTTCGGGCATATTGGAGTC
	TaqMan prove	CGTGCCCTTCGCGCTGACC
NT-3	Forward primer	GAGGCACCCAGAGAACCAGA
	Reverse primer	TTGCAATCATCGGCTGGA
	TaqMan prove	CAGGGAGAGGCCACCAGGTCAGAA

Forward primer: 5′ to 3′; Reverse primer: 5′ to 3′; TaqMan probe: 5′ [FAM] to 3′ [TAMRA].

removed rapidly, immediately frozen, and stored at −70°C.

Four groups of both juvenile and adult rats [subjected to neonatal isolation followed by SRS (NI + SRS); neonatal isolation alone (NI); SRS alone (SRS); or sham treatment (Sham)] were used for real-time quantitative PCR. No more than two rats per experimental group were from any single litter. A total of 24 juvenile and 51 adult rats were subjected to real-time quantitative PCR analysis and a different set of rats was used for each experiment (real-time quantitative PCR and hybridization).

Real-time quantitative PCR

Total RNA was extracted with an RNAqueous Phenol-free Total RNA Isolation Kit (Ambion, Austin, TX). After treatment with RNase-free DNase I (Takara, Kusatsu, Japan), real-time quantitative PCR was performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) to quantify relative mRNA levels in samples. Real-time quantitative PCR was performed to amplify the genes encoding NGF, GDNF, and NT-3. The primers and TaqMan hybridization probe were designed using Primer Express software (Applied Biosystems). Table I shows the sequences and associated fluorescent dye of each PCR primers and TaqMan probe. The TaqMan probes, which were designed to hybridize to the PCR products, were labeled with a fluorescent reporter dye at the 5′ end and a quenching dye at the 3′ end. PCR was carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems). All standards and samples were assayed in triplicate. Each plate contained the same standard. Thermal cycling was initiated with an initial denaturation at 50°C for 2 min and 95°C for 10 min. After this initial step, 40 cycles of PCR (heating at 95°C for 15 s and 60°C for 1 min) were performed. The PCR assay for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using TaqMan Rodent GAPDH Control Reagents (Applied Biosystems). The PCR assays on the test samples were performed simultaneously with the assays on the standard samples (rat brain tissue), which allowed us to construct a standard curve. The estimated relative expression of GAPDH and NGF, GDNF, or NT-3 mRNAs in the test samples were calculated using this standard curve and the ratio of the relative expression of NGF, GDNF, or NT-3 mRNAs was calculated relative to the expression of GAPDH.

Hybridization paradigm

Analysis of NGF and GDNF expression by in situ hybridization was conducted according to the method of Wetmore et al. (1992) with a minor modification. Oligonucleotide probes complementary to parts of the mRNAs encoding NGF and GDNF were synthesized and labeled with DIG at the 5′ end. The following antisense DNA oligonucleotides were used: NGF: 5′-CTG CGG GCT CTG CGG AGG GCT GTG TCA AGG GAA TGC TGA AGT TTA GTC CA-3′ (Nosrat et al., 1997) and GDNF: 5′-AGC CAC CAT CAA AAG ACT GAA AAG GTC ACC AGA TAA ACA AGC GGC GGC A-3′ (Lin et al., 1993).

Fresh-frozen coronal brain sections (15 μm) through the hippocampus were prepared using a cryostat, thaw-mounted onto slides (Matsunami, Kishiwada, Japan) and fixed with 4% paraformaldehyde for 5 min. Sections were hybridized for 18 h at 42°C in a humidified chamber with at least 0.5 ng probe/slide in a mixture of 4 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate), 50% formamide, 1 × Denhardt's solution (0.02% each of polyvinylpyrrolidone, bovine serum albumin, and Ficoll), 1% sarcosyl, 0.02 M phosphate buffer (pH 7.0), 10% dextran sulfate, 1 mg/ml of yeast RNA, and 200 mM dithiothreitol. Following hybridization, slides were rinsed for 5 × 15 min at 55°C in 2 × SSC, and allowed to reach room temperature. The sections were then incubated at 4°C overnight with an antidigoxigenin-alkaline phosphatase conjugate (Roche, Basel, Switzerland). After the incubation, the sections were extensively washed in PBT containing 0.2% BSA and 4 mM Levamisol, then washed in the reaction buffer (100 mM NaCl, 100 mM TrisHCl at pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20, and 4 mM Levamisol), and incubated with chromagen containing nitroblue tetrazolium salt (337 mg/ml) and 5-bromo-4-chloro-3-indoyl phosphate (175 mg/ml) according to the manufacturer's protocol (Roche). After color development (DIG labeling is visualized as a blue reaction product under light microscopy), slides were photographed under light microscopy (Model BZ-8000) (Keyence, Osaka, Japan). The mean density of sections was measured in the CA1 and CA3 regions (400 μm × 400 μm), and granule cell layer of the DG (400 μm × 200 μm) using the NIH Scion Image analysis program (Figs. 3a and 4a).

Statistical analysis

Results were expressed as mean ± SEM. The results of real-time quantitative PCR were analyzed

by two-way analysis of variance (ANOVA) (neonatal isolation × single immobilization stress), and posthoc comparisons were performed using Scheffe's test. For hybridization analyses, the results of experiments containing two groups of rats were analyzed by the Mann-Whitney U test. Significance was set at $P < 0.05$.

RESULTS

We first performed real-time quantitative PCR analysis to measure NGF, GDNF, and NT-3 mRNA in RNA isolated from the hippocampus of juvenile and adult rats subjected to either neonatal isolation followed by SIS (NI + SIS), neonatal isolation alone (NI), SIS alone (SIS) or sham treatment. In both juvenile and adult rats, two-way ANOVA showed no significant effect of NI [juvenile: $F(1, 23) = 2.652$, $P = 0.119$; adult: $F(1, 23) = 4.257$, $P = 0.051$] on the level of NGF mRNA, but revealed a significant effect of SIS [juvenile: $F(1, 23) = 7.093$, $P = 0.015$; adult: $F(1, 23) = 4.926$, $P = 0.037$], and a significant interaction between NI and SIS [juvenile: $F(1, 23) = 12.437$, $P = 0.002$; adult: $F(1, 23) = 6.605$, $P = 0.017$] (Figs. 1a and 2a). Posthoc analysis revealed that the levels of NGF mRNA in the SIS group was significantly higher ($P < 0.05$) than those in the Sham or NI + SIS group for both juveniles and adults (Figs. 1a and 2a). However, there was no significant difference between the level of NGF mRNA in the NI + SIS and Sham groups. In situ hybridization analysis demonstrated that the level of NGF mRNA in the CA3 pyramidal cell layer ($P = 0.022$) and the DG granular cell layer ($P = 0.002$) of the NI + SIS group was significantly lower than that in the SIS group in adulthood (Figs. 3a and 3b).

In the analyses of GDNF mRNA in juvenile rats, we observed no significant effect of SIS on the level of GDNF mRNA [$F(1, 23) = 0.877$, $P = 0.360$], but we did observe a significant effect of NI [$F(1, 23) = 8.865$, $P = 0.007$]. We found the trend toward significant interaction between NI and SIS [$F(1, 23) = 4.328$, $P = 0.051$] (Fig. 1b). Posthoc analysis revealed that the level of GDNF mRNA ($P < 0.05$) in the NI + SIS group was significantly lower than that in the SIS group (Fig. 1b). In adulthood, we observed no significant effect of NI on the level of GDNF mRNA [$F(1, 23) = 0.017$, $P = 0.935$], but we did observe a significant effect of SIS [$F(1, 23) = 8.172$, $P = 0.009$], as well as a significant interaction between NI and SIS [$F(1, 23) = 12.143$, $P = 0.002$] (Fig. 2b). Posthoc analysis revealed that the level of GDNF mRNA ($P < 0.05$) in the NI + SIS group was significantly lower than that in the NI group (Fig. 2b). The levels of GDNF mRNA in the Sham group also tended to be lower than that in the NI group, although the difference did not reach statistical significance ($P = 0.096$).

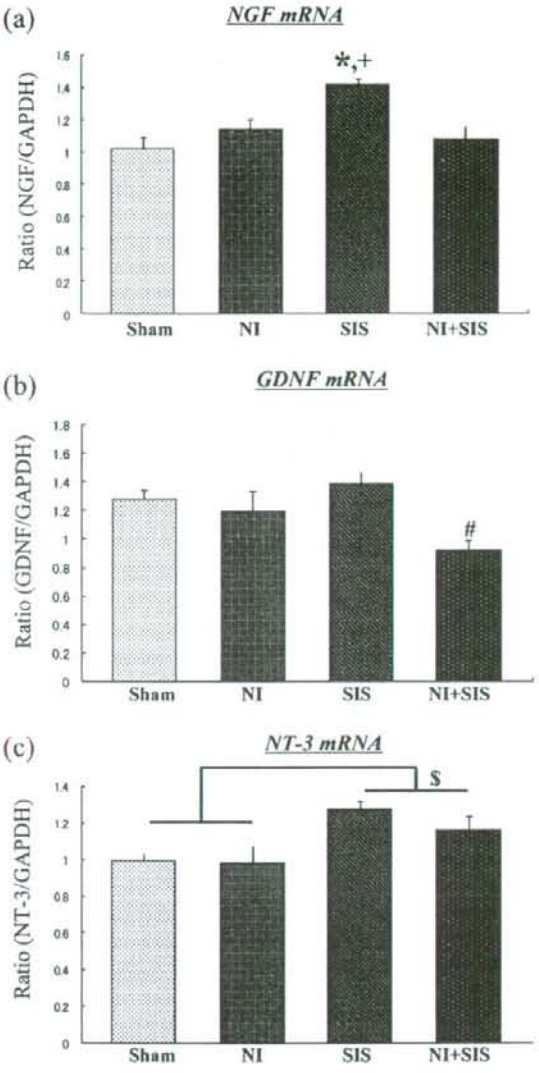

Fig. 1. Expression of (a) NGF, (b) GDNF, and (c) NT-3 mRNA in the hippocampus of juvenile rats subjected to sham treatment (Sham), neonatal isolation alone (NI), single immobilization stress alone (SIS), and neonatal isolation followed by a single immobilization stress (NI + SIS). Results are expressed as the ratio of the concentration of the target neurotrophin to that of GAPDH (target neurotrophin/GAPDH) percentage of Sham levels. The mean ± SEM ($n = 6$) is shown. $^{*}P < 0.05$ compared with sham, $^{\#}P < 0.05$ compared with SIS, $^{+}P < 0.05$ compared with NI + SIS, $^{\$}P < 0.05$ compared with Sham and NI (two-way ANOVA followed by Scheffe's test).

In situ hybridization analysis demonstrated that the level of GDNF mRNA in the CA1 ($P = 0.004$) and CA3 ($P = 0.025$) pyramidal cell layers and the DG ($P = 0.010$) granular cell layer of the NI + SIS group was significantly lower than that in the NI group (Fig. 4).

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