Table 2. Change in Pittsburgh Sleep Quality Index Score and Other Sleep Variables in Schizophrenia Patients Switched From Typical to Atypical Antipsychotic Treatment[a]

Variable	Baseline	Second Assessment	t Score	p Value[b]
Total score	8.58 ± 3.80	7.20 ± 4.29	3.246	.002
Subscores				
Sleep quality	1.12 ± 0.76	0.87 ± 0.87	2.305	.023
Sleep latency	1.72 ± 1.26	1.31 ± 1.26	3.090	.003
Sleep duration	0.60 ± 0.90	0.54 ± 0.94	0.565	.574
Habitual sleep efficiency	0.88 ± 1.06	0.56 ± 0.97	2.504	.014
Sleep disturbances	0.82 ± 0.53	0.69 ± 0.49	2.009	.047
Use of sleep medications	2.28 ± 1.26	2.27 ± 1.29	0.695	.488
Daytime dysfunction	1.16 ± 0.64	0.97 ± 0.59	2.473	.015
Other sleep variables				
Time in bed, min	540.1 ± 53.2	516.0 ± 47.9	3.667	.004
Total sleep time, min	441.5 ± 89.1	446.5 ± 86.3	0.477	.634
Sleep latency, min	54.7 ± 58.1	38.4 ± 50.2	2.410	.018
Sleep efficiency, %	81.8 ± 14.9	86.5 ± 14.6	2.541	.012

[a]Values are mean ± SD.
[b]p Values were obtained with paired t test.

chotics were discontinued over a period of 4 weeks. During this tapering period, 1 of the 4 atypical antipsychotic drugs was started, and its dosage was increased gradually. The target dose of atypical antipsychotic drugs was determined from the dose of ongoing typical antipsychotic drugs. After completion of switching, a psychiatrist adjusted dosages depending on the subject's clinical status. During the study, the antipsychotic dose was allowed to vary within the following ranges: olanzapine, 2.5 to 20.0 mg/day; perospirone, 4.0 to 48.0 mg/day; quetiapine, 50.0 to 750.0 mg/day; and risperidone, 1.0 to 12.0 mg/day. Mean dose levels at endpoint were 16.5 mg/day for olanzapine, 37.3 mg/day for perospirone, 432.5 mg/day for quetiapine, and 7.4 mg/day for risperidone.

Sixty-three percent of the subjects received hypnotics, 29% received anxiolytics, 20% received mood stabilizers, and 4% received antidepressants. These concomitant medications were not changed during the study.

Assessment of Quality of Sleep and Psychopathology

Subjective sleep quality and psychopathology were assessed at 2 intervals: baseline and 8 weeks after the switching was completed. We used the PSQI[19] to assess subjective sleep quality. The PSQI is a self-administered questionnaire to assess subjective sleep quality during the previous month. The self-rated items on the PSQI generate 7 subscale scores, each ranging from 0 to 3: sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbance, use of sleep medication, and daytime dysfunction. The sum of these 7 component scores yields 1 global score of subjective sleep quality ranging from 0 to 21, with higher scores representing poorer subjective sleep quality. The Positive and Negative Syndrome Scale (PANSS)[20] was used to rate schizophrenia symptoms. Data were collected from June 2001 to December 2001.

Data Analysis

Before analyzing sleep quality data, we examined demographic variables of each treatment group with analyses of variance (ANOVA) and the χ^2 test. The PSQI total score and each subscale score of the PSQI were compared before and after switching to atypical antipsychotic drugs. These comparisons were performed using a paired t test for all subjects. One-way ANOVA was conducted to compare the PSQI change scores in the 4 medication groups. The Fisher protected least significant difference test was performed for post hoc comparison. Multiple regression analysis was used to investigate the factors that predict improvement of sleep quality with atypical antipsychotic drug treatment. Change in PSQI total scores was entered into the analysis as a dependent variable. Demographic variables, the total PANSS score, subjective sleep quality, and the chlorpromazine-equivalent dosage of antipsychotic drugs at baseline were entered as independent variables for all subjects. The relationships between the PSQI total scores and the PANSS positive, negative, general, and total scores were investigated by Pearson correlation analysis. In addition, the relationships between changes in PSQI total scores and changes in PANSS positive, negative, general, and total scores were investigated. The results are expressed as means ± standard deviations, and significance was set at $p < .05$.

RESULTS

Demographic and clinical characteristics of the 92 patients are shown in Table 1. No statistically significant difference was detected among the 4 groups in age, distribution by sex, or duration of illness. Neither were there differences in baseline severity of psychopathology as assessed by the PANSS, subjective sleep quality as assessed by the PSQI, or chlorpromazine-equivalent dosages of antipsychotic drugs. Of the patients treated with perospirone, 1 dropped out before the second assessment because of worsening of psychotic symptoms. Of the patients treated with quetiapine, 1 dropped out before the second assessment because of hip fracture. All patients treated with olanzapine and risperidone completed the study protocol.

Changes in the PSQI total score and each subscale score with administration of the atypical antipsychotic drugs are shown in Table 2. Compared with conventional antipsychotic drugs, subjective sleep quality as assessed using the PSQI total score was significantly improved. As for the PSQI subscores, sleep quality, sleep latency, habitual sleep efficiency, sleep disturbance, and daytime dysfunction were improved, but sleep duration and use of sleep medication did not change in the total patient popu-

Table 3. Pittsburgh Sleep Quality Index Change in Score in Schizophrenia Patients Switched From Typical to Atypical Antipsychotics

Score	Olanzapine	Perospirone	Quetiapine	Risperidone	F Value	p Value[a]
Total	−3.20 ± 3.95**	1.56 ± 4.01	−1.93 ± 4.19**	−2.45 ± 2.89**	6.714	.0004
Subscale						
Sleep quality	−0.50 ± 1.15	0.22 ± 0.85	−0.33 ± 0.87	−0.35 ± 0.88	2.521	.063
Sleep latency	−0.45 ± 1.43	−0.22 ± 1.17	−0.59 ± 1.28	−0.35 ± 1.23	0.380	.76
Sleep duration	−0.55 ± 1.10**	0.69 ± 1.14	−0.22 ± 0.93**	−0.25 ± 0.97**	6.018	.0009
Habitual sleep efficiency	−0.80 ± 1.11**	0.47 ± 1.20	−0.44 ± 1.28**	−0.65 ± 1.18**	5.212	.0024
Sleep disturbances	−0.20 ± 0.62	0.04 ± 0.56	−0.11 ± 0.42	−0.25 ± 0.71	1.086	.36
Use of sleep medications	−0.05 ± 0.22	0.13 ± 1.10	−0.07 ± 1.04	−0.30 ± 0.92	0.796	.50
Daytime dysfunction	−0.65 ± 0.75**	0.21 ± 0.67	−0.15 ± 0.82*	−0.30 ± 0.57*	5.418	.0018

[a]p Values were obtained with analyses of variance. Fisher test was performed for post hoc comparison.
*p < .05 compared with perospirone.
**p < .01 compared with perospirone with Fisher test.

Table 4. Multiple Regression Analysis of Factors Predicting Improvement of PSQI Score in Schizophrenia Patients Switched From Typical to Atypical Antipsychotics[a]

Variable	Coefficient	SE	Standardized Coefficient	t Score	p Value
Sex	0.242	0.929	0.029	0.260	.79
Age	−0.018	0.055	−0.046	0.329	.74
Illness duration	0.003	0.005	0.078	0.574	.56
Schizophrenia type (undifferentiated)[b]					
Disorganized	−0.609	1.029	−0.068	0.592	.56
Paranoid	0.528	1.385	0.041	0.381	.70
Baseline antipsychotic drugs[c]	0.0002	0.0004	0.046	0.429	.67
Baseline PANSS total score	0.012	0.032	0.044	0.380	.71
Baseline PSQI total score	−0.462	0.118	−0.423	3.923	.0002

[a]$R^2 = 0.194$; adjusted $R^2 = 0.115$.
[b]For the categorical variables, the first type (undifferentiated) was used as the reference category.
[c]Converted to chlorpromazine equivalent dosage.
Abbreviations: PANSS = Positive and Negative Syndrome Scale, PSQI = Pittsburgh Sleep Quality Index.

lation. Some actual sleep variables are shown in Table 2. Time in bed and sleep latency were shortened, and sleep efficiency was improved with administration of the atypical antipsychotic drugs.

An overall difference in efficacy among the 4 atypical antipsychotic drugs was observed (Table 3). Olanzapine, risperidone, and quetiapine were superior to perospirone in their improvement of the PSQI total score, sleep duration, habitual sleep efficiency, and daytime dysfunction.

Multiple regression analysis revealed that the improvement of subjective sleep quality with administration of atypical antipsychotic drugs was predicted by poor sleep quality at baseline (Table 4). The correlation between subjective sleep quality and the severity of psychotic symptoms was insignificant at baseline (Table 5); however, the improvement of subjective sleep quality was significantly correlated with improvement of negative symptoms as assessed by the PANSS (Table 6).

DISCUSSION

While there are several reports on the effect of atypical antipsychotic drugs on sleep quality in patients with schizophrenia, to our knowledge, no previous report has compared the effect of atypical antipsychotic drugs on sleep quality with that of typical conventional antipsychotic drugs in the same group of patients with schizophrenia. This appears to be the first such study.

The present study demonstrated that, when compared with conventional antipsychotic drugs, subjective sleep quality as assessed by the PSQI was significantly improved in the study patients with schizophrenia after administration of olanzapine, risperidone, or quetiapine. No improvement was noted with perospirone. Also, improvement of subjective sleep quality after change in treatment was predicted by poor sleep quality at baseline and also was significantly correlated with improvement of negative scores on the PANSS.

The reason for the improvement of sleep disturbances with atypical antipsychotic drugs compared with typical conventional antipsychotic drugs has been postulated to be due to the higher potency of 5-HT_2 receptor blockade. It has been reported that a selective 5-HT_2 (5-HT_{2A} < 5-HT_{2C}) receptor antagonist, ritanserin, increases the duration of SWS in rats,[21,22] healthy volunteers,[9,10] and patients with dysthymia.[11] Olanzapine has a high affinity for both 5-HT_{2A} and 5-HT_{2C} receptors and caused an increase of SWS in healthy volunteers[15] and in drug-free patients with schizophrenia.[14] In contrast, the 5-HT_{2C} agonist, meta-chlorophenylpiperazine (m-CPP), was reported to decrease SWS in healthy volunteers.[23,24] The amount of SWS and sleep continuity predict subjec-

Table 5. Correlations Between PSQI Total Scores and PANSS Scores at Baseline in Schizophrenia Patients Switched From Typical to Atypical Antipsychotics

	PSQI Total Scores	
PANSS Scores	r Value	p Value[a]
Positive	0.120	.253
Negative	–0.074	.485
General	0.084	.424
Total	0.058	.584

[a]p Values were obtained with Pearson correlation analysis.
Abbreviations: PANSS = Positive and Negative Syndrome Scale, PSQI = Pittsburgh Sleep Quality Index.

Table 6. Correlations Between Change in PSQI Total Scores and Change in PANSS Scores (baseline to follow-up) in Schizophrenia Patients Switched From Typical to Atypical Antipsychotics

	Change in PSQI Total Scores	
Change in PANSS Scores	r Value	p Value[a]
Positive	0.200	.083
Negative	0.254	.026
General	0.001	.999
Total	0.147	.206

[a]p Values were obtained with Pearson correlation analysis.
Abbreviations: PANSS = Positive and Negative Syndrome Scale, PSQI = Pittsburgh Sleep Quality Index.

tive quality of sleep.[25,26] In this context, it is conceivable that the blockade of 5-HT_2 receptors may be associated with the regulation of sleep quality by increasing SWS. These findings suggest that the potent 5-HT_2 receptor blockade may be closely involved in the improvement of sleep quality shown in this study.

Another possible explanation is that the difference in sedative effect between conventional and atypical antipsychotic drugs improved the subjective quality of sleep. Wirz-Justice and colleagues[27] recorded wrist activities in hospitalized schizophrenia patients. They found that changing the antipsychotic medication from haloperidol (conventional) to clozapine (atypical) improved rest/activity rhythm. They also found that patients taking atypical antipsychotic drugs showed a higher level of daytime activity than did patients taking conventional antipsychotic drugs.[28] It is conceivable that the decrease in sedating effect, which occurs when changing from conventional to atypical antipsychotic drugs, may induce more consolidated nighttime sleep and consequently improve sleep quality, as was found in our study.

Although all 4 of the atypical antipsychotic drugs used in the present study have high potency of 5-HT_2 receptor blockade, the effect of perospirone on subjective sleep quality differed from the others. The precise mechanism of this difference is not known, but it is plausible that a potent agonistic action on 5-HT_{1A} receptors by perospirone may be involved. Clozapine, the first atypical antipsychotic drug that appeared for clinical use, also has high 5-HT_{1A} receptor agonist affinity. Although clozapine improves psychotic symptoms in patients with schizophrenia,[29] its administration was reported to reduce the amount of SWS.[29–31] Similarly, the 5-HT_{1A} agonist, m-CPP, was reported to decrease SWS in healthy volunteers.[23,24] In addition, the increase in the amount of SWS correlated with the improvement of subjective sleep quality.[25,26] Taken together, these findings indicate that perospirone might deteriorate subjective sleep quality partly through a decrease of SWS by its 5-HT_{1A} receptor agonist affinity.

Our results also support early findings that the severity of psychotic symptoms may correlate with sleep quality. Kupfer and coworkers[32] reported that the waxing phase of psychosis was associated with decreased total sleep. Following antipsychotic drug treatment, clinical improvement was associated with shorter sleep latency and improvement of sleep efficiency.[33] In addition, minutes of SWS were inversely correlated with the severity of negative symptoms in never-medicated patients with schizophrenia.[5]

The correlation between subjective sleep quality and severity of psychotic symptoms was insignificant at baseline. However, changes in subjective sleep quality were significantly correlated with changes in negative symptom scores on the PANSS in the present study.

Although the results of the present study demonstrate that changing from typical to atypical antipsychotic drugs significantly improves both subjective quality of sleep and negative symptoms, the precise mechanism of this improvement is still unknown. Conventional antipsychotic drugs are generally more sedating than atypical antipsychotic drugs.[27,28] Sedated patients tend to become socially isolated. It is possible that the improvement in negative symptoms noted with administration of atypical antipsychotic drugs may be due to increased daytime alertness and activity as a result of improved sleep quality and a decreased sedative effect.

In summary, switching from conventional antipsychotic drugs to atypical antipsychotic drugs significantly improved subjective sleep quality as assessed by the PSQI. This improvement was significantly correlated with the improvement of psychotic symptoms as determined by negative scores on the PANSS. Atypical antipsychotic drugs may also be particularly beneficial for the treatment of patients with schizophrenia experiencing sleep disturbances.

When interpreting these findings, it should be considered that the limitations of this study are its lack of a crossover design, which may bias efficacy findings in favor of the atypical antipsychotic drugs, and the absence of polysomnographic measurements. Further controlled double-blind studies with a crossover design and the addition of polysomnographic measures are required to confirm these findings.

Drug names: chlorpromazine (Thorazine, Sonazine, and others), clozapine (Fazaclo, Clozaril, and others), haloperidol (Haldol and others), olanzapine (Zyprexa), quetiapine (Seroquel), risperidone (Risperdal).

REFERENCES

1. Tandon R, Shipley JE, Greden JF, et al. Electroencephalographic sleep abnormality in schizophrenia: relationship to positive/negative symptoms and prior neuroleptic treatment. Arch Gen Psychiatry 1992;49:185–194
2. Benson KL, Zarcone VP Jr. Rapid eye movement sleep and eye movements in schizophrenia and depression. Arch Gen Psychiatry 1993;50: 474–482
3. Zarcone VP Jr, Benson KL, Berger PA. Abnormal rapid eye movement latencies in schizophrenia. Arch Gen Psychiatry 1987;44:45–48
4. Orzack MH, Hartman EL, Kornetsky C. The relationship between attention and slow wave sleep in schizophrenia. Psychopharmacol Bull 1977;13:59–61
5. Ganguli R, Reynolds CF, Kupfer DJ. Electroencephalographic sleep in young, never-medicated schizophrenics: a comparison with delusional and nondelusional depressives and with healthy controls. Arch Gen Psychiatry 1987;44:36–44
6. Kaplan J, Dawson S, Vaughan T, et al. Effect of prolonged chlorpromazine administration on the sleep of chronic schizophrenics. Arch Gen Psychiatry 1974;31:62–66
7. Jus K, Beland C, Bouchard M, et al. Polygraphic night sleep pattern during chronic single and multiple neuroleptic dose administration. Int Pharmacopsychiatry 1975;10:58–63
8. Maixner S, Tandon R, Eiser A, et al. Effects of antipsychotic treatment on polysomnographic measures in schizophrenia: a replication and extension. Am J Psychiatry 1998;155:1600–1602
9. Idzikowski C, Mills FJ, Glennard R. 5-hydroxytryptamine-2-antagonist increases human slow wave sleep. Brain Res 1986;378:164–168
10. Declerk AC, Wauquier A, Van der Ham-Veltman PHM, et al. Increase in slow-wave sleep in human with the serotonin-5HT2 antagonist ritanserin (the first exploratory polygraphic study). Curr Ther Res Clin Exp 1987;41:427–432
11. Paiva T, Arriaga F, Wauquier A, et al. Effects of ritanserin on sleep disturbances of dysthymic patients. Psychopharmacology (Berl) 1988;96:395–399
12. Dursun SM, Patel JK, Burke JG, et al. Effects of typical antipsychotic drugs and risperidone on the quality of sleep in patients with schizophrenia: a pilot study. J Psychiatry Neurosci 1999;24:333–337
13. Yamashita H, Morinobu S, Yamawaki S, et al. Effect of risperidone on sleep in schizophrenia: a comparison with haloperidol. Psychiatry Res 2002;109:137–142
14. Salin-Pascual RJ, Herrera-Estrella M, Galicia-Polo L, et al. Olanzapine acute administration in schizophrenic patients increases delta sleep and sleep efficiency. Biol Psychiatry 1999;46:141–143
15. Sharpley AL, Vassallo CM, Cowen PJ. Olanzapine increases slow-wave sleep: evidence for blockade of central 5-HT(2C) receptors in vivo. Biol Psychiatry 2000;47:468–470
16. Lee JH, Woo JI, Meltzer HY. Effects of clozapine on sleep measures and sleep-associated changes in growth hormone and cortisol in patients with schizophrenia. Psychiatry Res 2001;103:157–166
17. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. Washington, DC: American Psychiatric Press; 1994
18. Murasaki M, Koyama T, Machiyama Y, et al. Clinical evaluation of a new antipsychotic, perospirone HCl, on schizophrenia: a comparative double-blind study with haloperidol. Clin Eval 1997;24:159–205
19. Buysse DJ, Reynolds CF III, Monk TH, et al. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. Psychiatry Res 1989;28:193–213
20. Kay SR, Fiszbein A, Opler LA. The Positive and Negative Syndrome Scale (PANSS) for schizophrenia. Schizophr Bull 1987;13:261–276
21. Dugovic C, Wauquier A, Leysen JE, et al. Functional role of 5-HT2 receptors in the regulation of sleep and wakefulness in the rat. Psychopharmacology (Berl) 1989;97:436–442
22. Davenne D, Dugovic C, Franc B, et al. Ontogeny of slow wave sleep. In: Wauquier A, Dugovic C, Radulovacki M, eds. Slow Wave Sleep: Physiological, Pathological and Functional Aspects. New York, NY: Raven Press; 1989:21–30
23. Lawlor BA, Newhouse PA, Balkin TJ, et al. A preliminary study of the effects of nighttime administration of the serotonin agonist, m-CPP, on sleep architecture and behavior in healthy volunteers. Biol Psychiatry 1991;29:281–286
24. Katsuda Y, Walsh AE, Ware CJ, et al. meta-Chlorophenylpiperazine decreases slow-wave sleep in humans. Biol Psychiatry 1993;33:49–51
25. Keklund G, Akerstedt T. Objective components of individual differences in subjective sleep quality. J Sleep Res 1997;6:217–220
26. Rotenberg VS. The estimation of sleep quality in different stages and cycles of sleep. J Sleep Res 1993;2:17–20
27. Wirz-Justice A, Cajochen C, Nussbaum P. A schizophrenic patient with an arrhythmic circadian rest-activity cycle. Psychiatry Res 1997;73:83–90
28. Wirz-Justice A, Haug HJ, Cajochen C. Disturbed circadian rest-activity cycles in schizophrenia patients: an effect of drugs? Schizophr Bull 2001;27:497–502
29. Hinze-Selch D, Mullington J, Orth A, et al. Effects of clozapine on sleep: a longitudinal study. Biol Psychiatry 1997;42:260–266
30. Touyz SW, Beumont PJ, Saayman GS, et al. A psychophysiological investigation of the short-term effects of clozapine upon sleep parameters of normal young adults. Biol Psychiatry 1977;12:801–822
31. Wetter TC, Lauer CJ, Gillich G, et al. The electroencephalographic sleep pattern in schizophrenic patients treated with clozapine or classical antipsychotic drugs. J Psychiatr Res 1996;30:411–419
32. Kupfer DJ, Wyatt RJ, Scott J, et al. Sleep disturbance in acute schizophrenic patients. Am J Psychiatry 1970;126:1213–1223
33. Taylor SF, Tandon R, Shipley JE, et al. Effect of neuroleptic treatment on polysomnographic measures in schizophrenia. Biol Psychiatry 1991; 30:904–912

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Ginseng enhances contextual fear conditioning and neurogenesis in rats

ChunXiang Qiao[a], Ryousuke Den[b], Koutaro Kudo[a], Kazuo Yamada[a], Keiko Takemoto[c], Henny Wati[a], Shigenobu Kanba[d,*]

[a]*Department of Neuropsychiatry, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Japan*
[b]*Department of Neuropsychiatry, Keio University School of Medicine, Tokyo, Japan*
[c]*Graduate School of Nursing, Faculty of Medicine, University of Yamanashi, Japan*
[d]*Department of Neuropsychiatry, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashiku, Fukuoka 812-8582, Japan*

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Abstract

Panax Ginseng is a commonly used galenical known to have an enhancing effect on learning. Neurogenesis in the hippocampus has been shown to be necessary for hippocampus/amygdala-dependent learning tasks. To investigate the role of Ginseng in neurogenesis and learning of rats, we administered both Ginseng and BrdU for five consecutive days. As a result, Ginseng increased the number of BrdU-positive cells in the dentate gyrus in a dose-dependent manner. Further, we administered one dose of BrdU after Ginseng treatment for five consecutive days, and the number of BrdU-positive cells did not increase significantly. However, when one dose of BrdU was given 1 day before the following five consecutive days of Ginseng treatment, the number of BrdU-positive cells markedly increased in the hippocampus. Therefore, it is likely that Ginseng enhances not proliferation but survival of newly generated neurons in the hippocampus. Second, we administered both Ginseng and BrdU to rats for five consecutive days. One day after the last Ginseng and BrdU co-administration, contextual fear conditioning (CFC) was conducted. Ginseng in a dose-dependent manner increased the % freezing time and the number of BrdU-positive cells in the dentate gyrus of rats that received CFC. Thus, an increase in CFC-related neurogenesis may be one mechanism of Ginseng's properties to enhance learning ability.

Keywords: Ginseng; Neurogenesis; Learning; Hippocampus; Dentate gyrus; Contextual fear conditioning

1. Introduction

The Ginseng root (Panax Ginseng) is a common constituent of a large number of traditional oriental medicines. Among its diverse effects on the central nervous system, Ginseng is known to improve learning and memory. Although some of the early studies reported that Ginseng extracts caused learning impairment rather than improvement (Saito et al., 1977, 1979), subsequent studies showed that Ginseng extracts improve performance in active and passive avoidance learning tasks (Lasarova et al., 1987; Petkov et al., 1990, 1992, 1993). This discrepancy may be due to the sedative effect of Ginseng (Koo, 1999), which is observed with acute administration of Ginseng. Those that reported memory impairment by Ginseng examined the acute, but not chronic effects of Ginseng.

Chronic administration of Ginseng extracts or some of its fractions is known to improve learning and memory in several different hippocampus/amygdala-dependent behavioral tasks (Chang et al., 1998; Jaenicke et al., 1991; Jin et al., 1999; Lyubimov et al., 1997; Ni et al., 1993; Nitta et al., 1995a, 1995b; Watanabe et al., 1990; Wen et al., 1996; Yoshimura et al., 1998; Zhao and McDaniel, 1998; Zhong et al., 1998). However, the molecular and cellular mechanisms by which these agents exert behavioral effects remain to be explored.

In recent years, neurogenesis in the subgranular layer of the hippocampus (Gould et al., 1999b) and subsequent

* Corresponding author. Tel.: +81 92 642 5620; fax: +81 92 642 5644.
E-mail address: skanba@npsych.med.kyushu-u.ac.jp (S. Kanba).

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enhancement of neurotract connections (Nakagawa et al., 2002b) have drawn attention as one of the molecular biological mechanisms of hippocampus/amygdala-dependent learning. The hippocampus is one of few brain regions where production of neurons occurs throughout the lifetime of animals, including humans. Gould et al. (1999a) reported that improvement of a hippocampus-dependent task, such as a spatial learning test, was associated with increase in survival of bromodeoxyuridine (BrdU)-positive cells in rats.

To clarify the mechanism of learning enhancement by Ginseng, we investigated the effects of Ginseng on contextual fear conditioning (CFC), a hippocampus/amygdala-dependent learning task, and on neurogenesis in the hippocampus of rats. Prior to this investigation, we examined the effect of Ginseng on the baseline number of BrdU-positive cells in the hippocampus. For analysis of the phenotype of BrdU-positive cells, double staining with BrdU, a thymidine analog that labels dividing cells in S-phase, and NeuN, a neuronal marker, was used.

2. Materials and methods

2.1. Animals and Ginseng treatment

All experiments were conducted using 60 adult male Wister/ST rats (SLC Japan, 9 weeks old, weighing 270–290 g). They were group-housed (5 rats/group, 12 h light/dark cycle) with ad libitum access to food and water. The animals were treated in accordance with the Guidelines for Animal Experimentation (of the Ethics Review Committee) of the Faculty of Medicine, University of Yamanashi.

For the first experiment, Ginseng powder (supplied by Tsumura Pharmaceutical Co., Tokyo, Japan) was administered orally at doses of 0, 100, and 200 mg/kg/day for five successive days via a gastric tube. Dried Ginseng powder was suspended in tap water just before the use. The group denoted as 0 mg/kg/day received water only. Although in some previous reports Ginseng was administered by intraperitoneal (Mook-Jung et al., 2001) or intracerebroventricular injection (Kim et al., 1998), we considered that oral administration of Ginseng was crucial, because in traditional medicine Ginseng has always been taken orally. In addition, no data are available regarding the disposition of Ginseng in the body, such as absorption and metabolism, and more importantly, biologically active forms are not well identified. Some herbs are known to be metabolized into active forms by intestinal bacteria and then absorbed to exert pharmacological actions (Hasegawa et al., 1996). Thus, non-oral administration of Ginseng may result in non-pharmacological artifacts.

2.2. Contextual fear conditioning

CFC was conducted according to the method of Silva et al. (1998). The CFC task was performed in a conditioning chamber housed in a sound-attenuating box during the light phase of the cycle. The conditioning chamber (28 cm (width) × 21 cm (height) × 22 cm (diameter)) was constructed of clear Plexiglas. The floor of the chamber was lined with 18 stainless steel bars (4 mm in diameter; 1.5 cm spacing), which formed a foot shock grid to deliver scrambled shocks produced by a stimulator. The foot shock was 2 s direct current of 0.75 mA and served as the aversive unconditioned stimulus. The sound-attenuating box (48 cm (width) × 48 cm (height) × 48 cm (diameter)) was provided with a 20 W houselight, and a ventilation fan supplying background white noise (74 dB) was located on the top of the box. A discrete tone conditioned stimulus (CS) was given as a general contextual stimulus. The tone cue (800 Hz, 20 s duration, 80 dB) was delivered by two speakers located in the lower corner of the sound-attenuating box.

Prior to the conditioning, all rats received 3 days of habituation, in which they were placed in the conditioning chamber for 1 min and returned to their home cages once a day. On the day of conditioning, the rats were placed in the conditioning chamber and allowed to explore for 3 min. A foot shock was delivered 18 s after the tone CS. The rats were then allowed to recover for 30 s in the conditioning chamber and returned to their home cages. Two hours later, the rats were again introduced into the conditioning chamber and were tested for a 5 min period, during which no tone CS was delivered. Behavior was evaluated in terms of total freezing time during a 5 min (% freezing time) stay in the conditioning chamber. Freezing behavior was defined as cessation of all but respiratory movement. The rats were sacrificed immediately after % freezing time was measured.

Rats that experienced footshock were allocated to the CFC group and rats that did not experience footshock to the no-CFC group. There were three subgroups in the CFC and no-CFC groups, each of which was treated with 0, 100, or 200 mg/kg/day Ginseng powder. There were five rats in each subgroup.

2.3. Open field locomotion test

To test whether % freezing time was influenced by the sedative effect of Ginseng or not, the open field locomotion test was performed at the end of the treatment period on the sixth day. This test was performed on the rats that were administered 0 or 200 mg/kg/day of Ginseng.

The open field is a 750 mm× 750 mm wooden arena, with 300 mm high walls surrounding the field, painted black on all inner surfaces. Thin white stripes are painted across the floor, dividing it into 25 quadratic blocks. The open field instrument was cleaned after each individual test session to prevent the next rat from being influenced by the odors deposited in the urine and feces of the previous rat.

The rat was placed in the area with its head pointing to a corner. An observer manually quantified the rat's spontaneous ambulatory locomotion in the horizontal plane by

scoring the number of squares entered (crossing into a different adjacent section with all four extremities), during 5 min.

2.4. Immunostaining

On the sixth day, immediately after CFC performance, the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg ip) and perfused transcardially with 350 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The rat brain was quickly removed, and then post-fixed for 24 h in paraformaldehyde. Then, 40 μm-thick frontal sections were cut on a cryostat and collected in PBS (0.1 M; pH 7.4). In accordance with the rat brain map of Paxinos and Watson (1986), 40 μm-thick free-floating sections were prepared, and 12 sections were collected at 160 μm intervals for staining. DNA denaturation was conducted by incubation for 30 min in 50% formamide/2 × SSC at 65 °C followed by several rinses in 2 × SSC. Sections were then incubated for 30 min in 2N HCl and 10 min in boric acid. After washing in PBS, sections were incubated in 3% H_2O_2 to block endogenous peroxidase for 10 min. After blocking with 10% normal goat serum (NGS), sections were incubated with anti-BrdU (1:1000 Haralan Sera Lab. OBT0030) for 24 h at 4 °C. Sections were then incubated for 1 h with secondary antibody (biotinylated goat, anti-rat IgG; Vector BA9400) followed by amplification with an avidin–biotin complex, before developing the color using DAB.

The same number of free-floating sections from the control and the Ginseng (200 mg/kg/day for 5 days)-treated animals ($n = 6$) surviving 4 weeks after administration of one dose of BrdU before Ginseng treatment was used for analysis of phenotypes. Double immunostaining procedures with fluorescent chromogens were used to evaluate the co-expression of BrdU with neuronal nuclear protein NeuN. In the double-labeling experiment, BrdU was visualized with Streptavidin-Texas Red (Amersham Pharmacia Biotech, 1:100), while the neuronal marker NeuN (Chemicon International Inc., MAB377; 1:500) was visualized with FITC (anti-mouse Ig, fluorescein-linked whole antibody 1:20). Fluorescent signals were viewed using a TCS4D confocal laser-scanning microscope. The emission signals of Texas Red and FITC were assigned to red and green, respectively.

2.5. Quantification of BrdU labeling

Every fourth section throughout the hippocampus was processed for BrdU immunohistochemical study. All BrdU-labeled cells in the dentate gyrus (granular cell layer) and the hilus were counted in each section. To distinguish single cells within clusters, all counts were performed at 400× and 1000× magnification under a light microscope (Olympus BX-60), omitting cells in the outermost focal plane.

A cell was counted as being in the subgranular zone (SGZ) of the dentate gyrus if it was touching or in the SGZ. Cells that were located more than two cells away from the SGZ were classified as hilar cells. The cell number was divided by the area of the dentate gyrus, and then the mean positive cell number per square millimeter was counted.

2.6. Protocol of BrdU administration

To investigate the overall effects of Ginseng on neurogenesis in the dentate gyrus of rats and its association with CFC performance, rats were orally administered both Ginseng (0, 100, 200 mg/kg/day) and BrdU (200 mg/kg/day) simultaneously for five consecutive days. On the sixth day, the animals were subjected to CFC test and then sacrificed for immunohistochemical study as described above (Fig. 1A).

BrdU is taken up into cells that are in the S-phase of DNA synthesis. The S-phase lasts for approximately 2 h (Packard et al., 1973). When one dose of BrdU is given, it is incorporated only into cells in the S-phase. At least one cell cycle is completed in 24 h by cells in the S-phase after the time of BrdU injection (Nowakowski et al., 1989). Therefore, if an animal is given one dose of BrdU and decapitated 2 h later for immunostaining, cells that have newly proliferated are observed. On the contrary, if an animal is given one dose of BrdU and is decapitated a few days later, cells that were produced a few days ago and are still surviving at the time of decapitation are observed. Accordingly, it is possible to differentiate newly proliferated cells from surviving cells by varying the time interval between BrdU administration and decapitation.

In our study, to determine the effects of Ginseng on cell survival, BrdU was given once to drug-naive rats, and Ginseng was given for the following 5 days. Then, the rats were sacrificed for immunohistochemical study (Fig. 1B). On the other hand, to determine the effects of Ginseng on cell proliferation, BrdU was given once on the sixth day, after Ginseng treatment for five consecutive days, and sacrificed 2 h after BrdU labeling (Fig. 1C).

2.7. Statistical analysis

All the results are presented as mean ± S.E.M. One-way analysis of variance (ANOVA) was used to examine the effects of CFC and Ginseng administration on % freezing time because of their interaction, since there was interaction between the two groups (Fig. 6). Two-way analysis of variance (ANOVA) was used to examine the effect of CFC and Ginseng on number of BrdU-positive cells in rats subjected to CFC (Fig. 2). As shown in Fig. 5, Student's *t*-test was used to determine whether the effect of Ginseng treatment on the number of BrdU-positive cells is related to cell proliferation or cell survival. Differences in the values of locomotion measured in the open field test were examined by Student's *t*-test.

A P-value < 0.05 was considered to indicate a statistically significant difference.

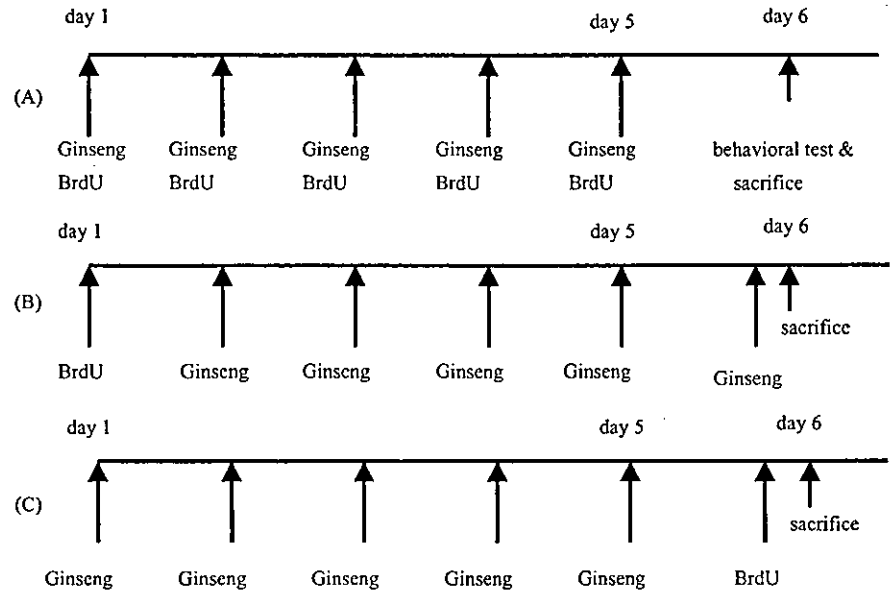

Fig. 1. Experimental procedures to examine effects of Ginseng on CFC-associated neurogenesis, cell survival and cell proliferation in rat hippocampus. Rats were orally administered both Ginseng (0, 100, 200 mg/kg/day) and BrdU (200 mg/kg/day) for five consecutive days. On the sixth day, the animals were subjected to CFC test (A). To determine the effects of Ginseng on cell survival, BrdU was given once to drug-naive rats, and Ginseng was given for the following 5 days. (B) To determine the effects of Ginseng on cell proliferation, BrdU was given once on the sixth day, after Ginseng treatment for five consecutive days, and sacrificed 2 h after BrdU labeling (C).

3. Results

3.1. Effect of Ginseng on baseline number of BrdU-positive cells

To test the effect of Ginseng on neurogenesis in rats, we counted the number of BrdU-positive cells in the dentate gyrus. The number of BrdU-positive cells increased in a dose-dependent manner in the Ginseng treatment group (Figs. 2A and 3).

3.2. Phenotype of BrdU-positive cells

The phenotype of the BrdU-positive cells in the granule cell layer was examined in the Ginseng group 4 weeks after BrdU labeling. We performed immunostaining for BrdU as

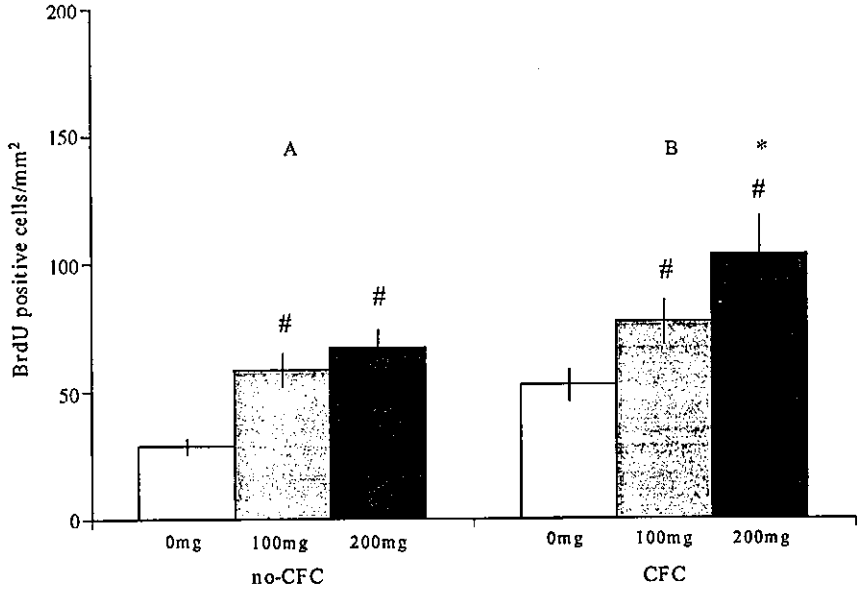

Fig. 2. Change in BrdU-positive cells in rat hippocampus after CFC performance. The number of BrdU-positive cells was higher in the CFC groups than in the no-CFC groups ($P = 0.0113$, $F_{1,24} = 13.718$). The number of BrdU-positive cells was significantly increased in a dose-dependent manner by Ginseng treatment, both in the no-CFC groups (A) and the CFC groups (B) ($P = 0.0001$, $F_{2,24} = 13.538$) (mean ± S.E.M.). (#) $P < 0.05$ vs. 0 mg; (*) $P < 0.05$ for no-CFC vs. CFC.

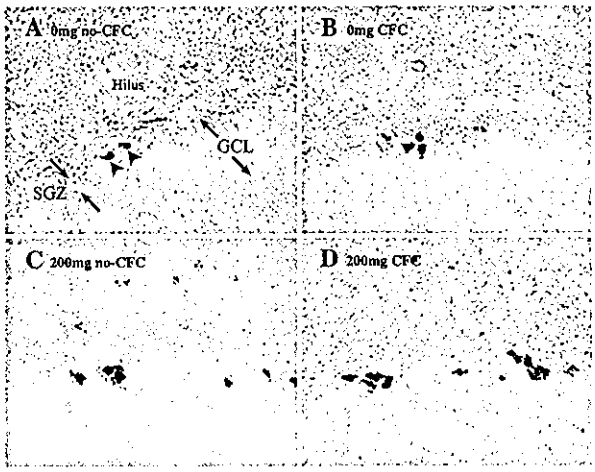

Fig. 3. Optical photomicrographs of changes in number of BrdU-positive cells in the hippocampus after CFC (40× magnification) BrdU-positive cells (arrowhead) were increased in the CFC groups (B and D) compared to the no-CFC groups (A and C). BrdU-positive cells were increased in a dose-dependent manner by Ginseng, in both the CFC groups and no-CFC groups. (A) Ginseng 0 mg and no-CFC; (B) Ginseng 0 mg and CFC; (C) Ginseng 200 mg and no-CFC; (D) Ginseng 200 mg and CFC. The majority of BrdU-positive cells were located in the subgranular zone (SGZ) of the hippocampus—the region between the granular cells layer (GCL) and hilus.

well as for NeuN, a marker for neurons (Fig. 4). It was found that ~80% of BrdU-positive cells expressed NeuN, and no significant difference existed in % NeuN-positive cells regardless of whether Ginseng was administered or not (data not shown).

3.3. Effect of Ginseng on cell survival

The increased neurogenesis may have been due to an increase in cell survival and/or cell proliferation. To investigate the effect of Ginseng specifically on cell survival, one dose of BrdU was given orally to the rats, followed by 5 days' administration of Ginseng (200 mg/kg/day). As a result, Ginseng did significantly increase the number of BrdU-positive cells. This finding showed that Ginseng enhanced the survival rate of newly generated cells in the hippocampus (see Fig. 5A).

3.4. Effect of Ginseng on cell proliferation

On the other hand, in order to investigate the effect of Ginseng on cell proliferation, Ginseng (200 mg/kg/day) was given for 5 days, and then one dose of BrdU was given to the rats 2 h before they were sacrificed for immunostaining. With this administration protocol, the number of BrdU-positive cells did not increase significantly in the dentate gyrus. This result suggested that the Ginseng employed in our experiment did not induce a significant change in cell proliferation in the hippocampus (see Fig. 5B).

3.5. Effect of Ginseng on % freezing time in CFC test and CFC-associated increase in BrdU-positive cells

As shown in Fig. 6A, in the rats that were not given foot shocks in the conditioning chamber (no-CFC group), Ginseng administration did not change % freezing time at any dose. CFC itself increased % freezing time. In the rat groups that received CFC (CFC groups), Ginseng treatment increased % freezing time in a dose-dependent manner (see Fig. 6B). Ginseng at 200 mg/kg significantly increased % freezing time as compared to Ginseng at 0 or 100 mg/kg. This result shows that Ginseng significantly enhances the performance of rats in the CFC, a hippocampus/amygdala-dependent learning task.

When Ginseng and BrdU were co-administered for five consecutive days, the number of BrdU-positive cells increased in a dose-dependent manner in the CFC groups, and the increase was significant at a dose of Ginseng of 200 mg/kg as compared to doses of 0 and 100 mg/kg (Figs. 2B and 3). CFC also increased the number of BrdU-positive cells as compared to the no-CFC groups, even when saline was co-administered with BrdU for 5 days (Figs. 2 and 3).

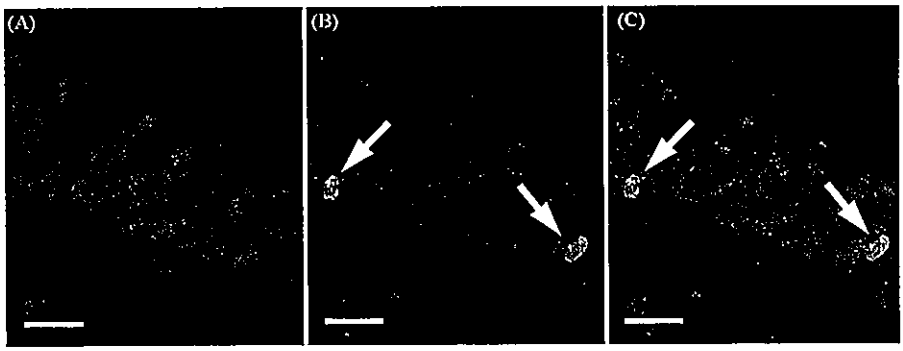

Fig. 4. Double immunolabeling of NeuN and BrdU in adult dentate gyrus of Ginseng-treated rats. Double immunolabeling of NeuN (green, A), a marker of mature neurons, and BrdU (red, B) in the adult dentate gyrus of Ginseng-treated rats surviving 4 weeks after BrdU administration. Confocal images (630× magnification) show NeuN immunoreactivity in BrdU-labeled cells (arrows, C) in both Ginseng-treated and non-treated animals. Scale bar: 20 μm.

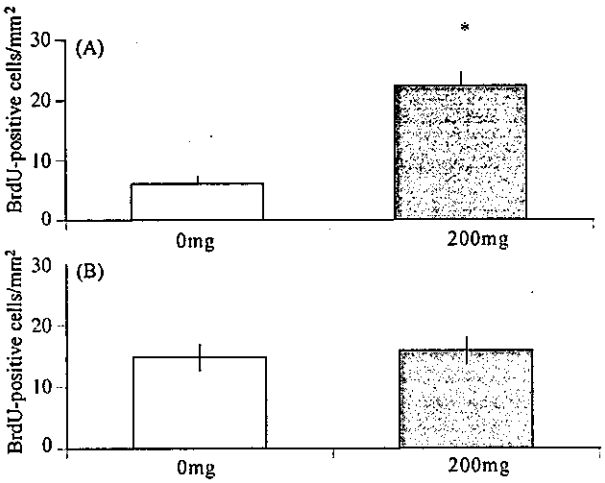

Fig. 5. Effects of Ginseng on BrdU-positive cells in rat hippocampus administration of Ginseng for five consecutive days after one treatment with BrdU significantly increased BrdU-positive cells compared to control rats ($n = 5$, $^{*}P = 0.0003$) (A). Administration of Ginseng for five consecutive days followed by one treatment with BrdU 24 h later caused no significant change in BrdU-positive cells compared to control rats ($n = 5$, $P = 0.7341$) (B).

3.6. Effect of Ginseng on open field locomotion

To examine whether % freezing time was influenced by the sedative effect of Ginseng, the open field test was performed. Total locomotion distance of the group treated with Ginseng 200 mg/kg/day for five consecutive days was not significantly different compared to the no Ginseng group (data not shown). There was no significant difference in grooming and rearing either (data not shown). This result indicates that Ginseng at 200 mg/kg did not cause any difference in spontaneous activity or locomotion compared with the non-treated rats.

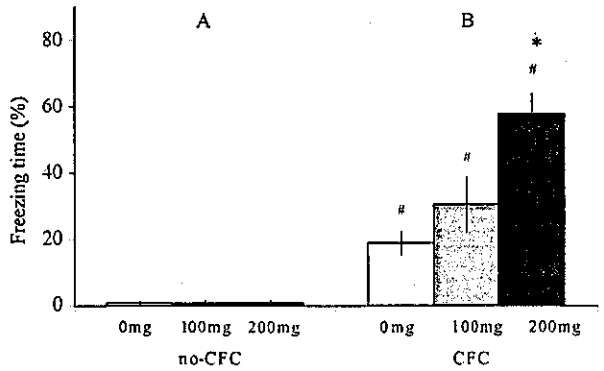

Fig. 6. Effects of Ginseng on % freezing time of rats tested in a conditioning chamber 1 day after finishing administration of Ginseng with BrdU for five consecutive days. Data are shown as mean ± S.E.M. There was a significant difference in % freezing time among the tested groups ($F_{5,24} = 25.312$, $P < 0.007$). (#) The 0, 100, and 200 mg Ginseng groups showed a significantly higher % freezing time in the CFC group than the no-CFC group, respectively. (*) The 200 mg/kg Ginseng group showed the highest % freezing time out of all the CFC groups (B). In contrast, the no-CFC groups did not show any change in % freezing time (A).

4. Discussion

Ginseng administration increased the number of BrdU-positive cells of the dentate gyrus in the no-CFC groups in a dose-dependent manner. Double staining with BrdU and NeuN suggested that the increase in BrdU-labeled cells may be mainly based on an increase in neurogenesis, and not gliogenesis.

Neurogenesis, defined as the creation of new nerve cells, consists of a series of distinct developmental steps, two of which can be examined separately; proliferation and survival/differentiation (Malberg et al., 2000). Our study suggested that the increased number of BrdU-positive cells induced by Ginseng was due to an increase in cell survival, and not cell proliferation.

We also found enhancing effects of Ginseng on performance in CFC, which is hippocampus/amygdala-dependent learning, as well as on neurogenesis in the dentate gyrus in the CFC groups. The unaltered result of the open field test in the Ginseng group shows that Ginseng causes neither a stimulating nor sedating effect at the doses employed in this study, which could potentially interfere with evaluation of CFC. Another interfering factor in evaluation of CFC is that Ginseng's effect on pain sensitivity. Since there is no previous data to suggest this effect, the possibility is low, but still remains to be examined.

Ginseng increased the CFC-related increase in the number of BrdU-positive cells in a dose-dependent manner. Although in this study we did not examine whether this CFC-related increase in the number of BrdU-positive cells was due to increased cell proliferation or cell survival, a previous study suggested that learning was associated with enhancement of cell survival but not cell proliferation (Fulder, 1981; Gould et al., 1999a), although it remains to be answered whether Ginseng and CFC have an additive or interactive effect on neurogenesis. It is reasonable to conclude that the increase in CFC-related neurogenesis may be one mechanism of Ginseng's property to enhance learning ability.

What are the molecular mechanisms underlying the regulation of hippocampal neurogenesis by Ginseng? Ginseng root consists of two major constituents: crude Ginseng saponin and crude Ginseng non-saponin fractions. To date, more than 20 saponins have been isolated from Ginseng root and identified chemically (Lim et al., 1997). Ginsenosides (the saponin constituents of Ginseng root) have been reported to have a number of actions on the CNS. These include CNS stimulation or depression (Watanabe et al., 1990), anticonvulsant activity (Gupta et al., 2001), anti-psychotic activity (Yoshimura et al., 1998), anti-fatigue (Wang et al., 1983) and anti-stress activity (Fulder, 1981; Kim et al., 1998), and improvement of performance in various memory paradigms (Jin et al., 1999; Ni et al., 1993; Nitta et al., 1995a).

The beneficial effects of Ginseng on learning and memory have often been attributed to ginsenoside Rb1

and Rg1 (Mook-Jung et al., 2001). Ginsenoside Rg1 increases cAMP level and c-fos gene expression in the rat hippocampus (Liu and Zhang, 1996). The elevation of intracellular cAMP level induces c-fos expression (Vaccarino et al., 1993). The cAMP–CREB cascade could contribute to the actions of neurotransmitters and neurotrophic factors on adult neurogenesis (Nakagawa et al., 2002a). In recent reports, CREB was shown to be necessary for both steps of neurogenesis: proliferation and cell survival (Nakagawa et al., 2002b). Since in our study, Ginseng did not enhance cell proliferation, activation of the cAMP-CREB cascade by Ginseng could not solely explain our findings.

Alternatively, the ginsenosides Rb1 and Rg1 (Mook-Jung et al., 2001) are also thought to enhance learning and memory by facilitation of cholinergic function, which is apparently essential for the functional integration of learning processes. For example, Rb1 facilitated acetylcholine (ACh) release and improved passive avoidance learning (Benishin et al., 1991). Rb1 and Rg1 increased the number of ACh receptors and improved passive avoidance learning in anisodine-treated mice (Shan et al., 2002). Rg1 improved the performance of scopolamine-injected rats in an eight-arm radial maze task. Consistent with these results, Rb1 facilitated choline uptake and increased choline acetyltransferase (Salim et al., 1997). ACh is also shown to increase neurogenesis (Ma et al., 2000). Thus, the increase of neurogenesis by Ginseng may be mediated via an increase in ACh release and ACh receptors.

In conclusion, Ginseng had an enhancing effect on CFC and increased the number of BrdU-positive cells in the dentate gyrus. The increase of neurogenesis by Ginseng was due to enhancement of cell survival, and not proliferation. Future study will be required to determine the components of Ginseng that are responsible for the enhancement of CFC and neurogenesis. Elucidation of the exact components and their mechanisms will lead to novel drugs for the treatment of memory impairment.

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References

Benishin, C.G., Lee, R., Wang, L.C., Liu, H.J., 1991. Effects of ginsenoside Rb1 on central cholinergic metabolism. Pharmacology 42, 223–229.

Chang, Y.S., Wu, C.R., Ho, Y.L., Hsieh, M.T., 1998. Effects of Panax Ginseng and its constituents on drug-induced memory impairment in rats. In: Proceedings of the Seventh International Symposium on Ginseng. pp. 289–299.

Fulder, S.J., 1981. Ginseng and the hypothalamic–pituitary control of stress. Am. J. Chin. Med. 6, 112–118.

Gould, E., Beylin, A., Tanapat, P., Reeves, A., Shors, T.J., 1999a. Learning enhances adult neurogenesis in the hippocampal formation. Nat. Neurosci. 2, 260–265.

Gould, E., Tanapat, P., Hastings, N., Shors, T.J., 1999b. Neurogenesis in adulthood: a possible role in learning. Trends Cog. Neurosci. 3, 186–192.

Gupta, Y.K., Sharma, M., Chaudhary, G., 2001. Antiepileptic activity of Panax Ginseng against pentylenetetrazole induced kindling in rats. Indian J. Physiol. Pharmacol. 45, 502–506.

Hasegawa, H., Sung, J.H., Matsumiya, S., Uchiyma, M., 1996. Main Ginseng saponin metabolites formed by intestinal bacteria. Planta Med. 62, 453–457.

Jaenicke, B., Kim, E.J., Ahn, J.W., Lee, H.S., 1991. Effect of Panax Ginseng extract on passive avoidance retention in old rats. Arch. Pharm. Res. 14, 25–29.

Jin, S.H., Park, J.K., Nam, K.Y., Park, S.N., Jung, N.P., 1999. Korean red Ginseng saponins with low ratios of protopanaxdiol and protopanaxatriol saponin improve scopolamine-induced learning disability and spatial working memory in mice. J. Ethnopharmacol. 166, 123–129.

Kim, D.H., Jung, J.S., Suh, H.W., Huh, S.O., Min, S.K., Son, B.K., Park, J.H., Kim, N.D., Kim, Y.H., Song, D.K., 1998. Inhibition of stress-induced plasma corticosterone levels by ginsenosides in mice: involvement of nitric oxide. Neuroreport 9, 2261–2264.

Koo, M.W., 1999. Effects of Ginseng on ethanol induced sedation in mice. Life Sci. 64, 153–160.

Lasarova, M.B., Mosharrof, A.H., Petkov, V.D., Markovska, V.L., Petkov, V.V., 1987. Effect of piracetam and of standardized Ginseng extracts on the electroconvulsive shock-induced memory disturbances in "step-down" passive avoidance. Acta Physiol. Pharmacol. Bulg. 13, 11–17.

Lim, J.H., Wen, T.C., Matsuda, S., Tanaka, J., Maeda, N., Peng, H., Aburaya, J., Ishihara, K., Sakanaka, M., 1997. Protection of ischemic hippocampal neurons by ginsenoside Rb1, a main ingredient of Ginseng root. Neurosci. Res. 28, 191–200.

Liu, M., Zhang, J.T., 1996. Effects of ginsenoside Rg1 on c-fos gene expression and cAMP levels in rat hippocampus. Zhongguo Yao Li Xue Bao 17, 171–174.

Lyubimov, I.I., Borzenkov, V.M., Chepurnova, N.E., Epurnova, S., 1997. Effect of a polysaccharide fraction of Ginseng root on learning and memory in rats. Neurosci. Behav. Physiol. 27, 555–558.

Ma, W., Maric, D., Li, B.S., Hu, Q., Andreadis, J.D., Grant, G.M., Liu, Q.Y., Shaffer, K.M., Chang, Y.H., Zhang, L., Pancrazio, J.J., Pant, H.C., Stenger, D.A., Barker, J.L., 2000. Acetylcholine stimulates cortical precursor cell proliferation in vitro via muscarinic receptor activation and MAP kinase phosphorylation. Eur. J. Neurosci. 4, 1227–1240.

Malberg, J.E., Eisch, A.J., Nestler, E.J., Duman, R.S., 2000. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J. Neurosci. 24, 9104–9110.

Mook-Jung, I., Hong, H.S., Boo, J.H., Lee, K.H., Yun, S.H., Cheong, M.Y., Joo, I., Huh, K., Jung, M.W., 2001. Ginsenoside Rb1 and Rg1 improve spatial learning and increase hippocampal synaptophysin level in mice. J. Neurosci. Res. 1563, 509–515.

Nakagawa, S., Kim, J.E., Lee, R., Malberg, J.E., Chen, J., Steffen, C., Zhang, Y.J., Nestler, E.J., Duman, R.S., 2002a. Regulation of neurogenesis in adult mouse hippocampus by cAMP and cAMP response element-binding protein. J. Neurosci. 22, 3673–3682.

Nakagawa, S., Kim, J.E., Lee, R., Chen, J., Fujioka, T., Malberg, J., Tsuji, S., Duman, R.S., 2002b. Localization of phosphorylated cAMP response element-binding protein in immature neurons of adult hippocampus. J. Neurosci. 22, 9868–9876.

Ni, X.H., Ohta, H., Watanabe, H., Matsumoto, K., 1993. Panax Ginseng extract improves scopolamine-induced deficits in working memory

performance in the T-maze delayed alternation task in rats. Phytother. Res. 7, 49–52.

Nitta, H., Matsumoto, K., Shimizu, M., Ni, X.H., Watanabe, H., 1995a. Panax Ginseng extract improves the performance of aged Fischer 344 rats in radial maze task but not in operant brightness discrimination task. Biol. Pharm. Bull. 18, 1286–1288.

Nitta, H., Matsumoto, K., Shimizu, M., Ni, X.-H., Watanabe, H., 1995b. Panax Ginseng extract improves the scopolamine-induced disruption of 8-arm radial maze performance in rats. Biol. Pharm. Bull. 18, 1439–1442.

Nowakowski, R.S., Lewin, S.B., Miller, M.W., 1989. Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J. Neurocytol. 18, 311–318.

Packard Jr., D.S., Menzies, R.A., Skalko, R.G., 1973. Incorporation of thymidine and its analogue, bromodeoxyuridine, into embryos and maternal tissues of the mouse. Differentiation 1, 397–404.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates. Academic Press, San Diego.

Petkov, V.D., Mosharrof, A.H., Petkov, V.V., Kehayov, R.A., 1990. Age-related differences in memory and in the memory effects of nootropic drugs. Acta Physiol. Pharmacol. Bulg. 16, 28–36.

Petkov, V.D., Cao, Y., Todorov, I., Lazarova, M., Getova, D., Stancheva, S., Alova, L., 1992. Behavioral effects of stem-leaves extracts from Panax Ginseng C.A. Meyer. Acta Physiol. Pharmacol. Bulg. 18, 41–48.

Petkov, V.D., Kehayov, R., Belcheva, S., Konstantinova, E., Petkov, V.V., Getova, D., Markovska, V., 1993. Memory effects of standardized extracts of Panax Ginseng (G115) Ginkgo biloba (GK501) and their combination Gincosan (PHL-00701). Planta Med. 59, 106–114.

Saito, H., Tsuchiya, M., Naka, S., Takagi, K., 1977. Effects of Panax Ginseng root on conditioned avoidance response in rats. Jpn. J. Pharmacol. 27, 509–516.

Saito, H., Tsuchiya, M., Naka, S., Takagi, K., 1979. Effects of Panax Ginseng root on acquisition of sound discrimination behavior in rats. Jpn. J. Pharmacol. 29, 319–324.

Salim, K.N., McEwen, B.S., Chao, H.M., 1997. Ginsenoside Rb1 regulates ChAT, NGF and trkA mRNA expression in the rat brain.. Brain Res. Mol. Brain Res. 47, 177–182.

Shan, S.J., Xu, Q.P., Shoyama, Y., 2002. Extract of Yi Zhi Fang improves learning and memory behaviours of mice and its possible mechanisms. Phytother. Res. 5, 449–454.

Silva, A.J., Kogan, J.H., Frankland, P.W., Kida, S., 1998. CREB and memory. Annu. Rev. Neurosci. 21, 127–148.

Vaccarino, F.M., Hayward, M.D., Le, H.N., Hartigan, D.J., Duman, R.S., Nestler, E.J., 1993. Induction of immediate early genes by cyclic AMP in primary cultures of neurons from rat cerebral cortex.. Brain Res. Mol. Brain Res. 19, 76–82.

Wang, B.X., Cui, J.C., Liu, A.J., Wu, S.K., 1983. Studies on the anti-fatigue effect of the saponins of stems and leaves of Panax Ginseng (SSLG). J. Tradit. Chin. Med. 3, 89–94.

Watanabe, H., Ohta, H., Matsumoto, K., 1990. Effect of Ginseng on spontaneous motor activity, water maze learning and central dopaminergic systems in old rats. In: Shibata, S., Ohtsuka, Y., Saito, H. (Eds.), Recent Advances in Ginseng Studies. Tokyo Hirokawa, Tokyo, pp. 73–81.

Wen, T.C., Yoshimura, H., Matsuda, S., Lim, J.H., Sakanaka, M., 1996. Ginseng root prevents learning disability and neuronal loss in gerbils with 5-min forebrain ischemia. Acta Neuropathol. 91, 15–22.

Yoshimura, H., Watanabe, K., Ogawa, N., 1998. Psychotropic effects of Ginseng saponins on agonistic behavior between resident and intruder mice. Eur. J. Pharmacol. 146, 291–297.

Zhao, R., McDaniel, W.F., 1998. Ginseng improves strategic learning by normal and brain-damaged rats. Neuroreport 9, 1619–1624.

Zhong, Y.M., Nishijo, H., Uwano, T., Yamaguchi, H., Ono, T., 1998. Red Ginseng ameliorates place learning deficits in aged rats and in young rats with selective hippocampal lesions. In: Proceedings of the Seventh International Symposium on Ginseng. pp. 1–11.

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Two-dimensional neural activity mapping of the entire population of hippocampal CA1 pyramidal cells responding to fear conditioning

Koutarou Inoue[a,b,1], Yugo Fukazawa[a,2], Akihiko Ogura[b], Kaoru Inokuchi[a,*]

[a]*Mitsubishi Kagaku Institute of Life Sciences (MITILS), 11 Minamiooya, Machida, Tokyo 194-8511, Japan*
[b]*Department of Biology, Osaka University of Graduate School of Science, Toyonaka, Osaka 560-0043, Japan*

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Abstract

The hippocampus is involved in the encoding, storage, and retrieval of memory. Here, we have developed a novel mapping method for detecting the distribution of neural activity of the entire population of pyramidal cells in the hippocampal CA1 and subiculum regions, where expression profiles of *Arc* mRNA were used as an indicator of neural activity. The spherical pyramidal cell layer of the intact hippocampus was flattened into a two-dimensional plane, which was then serially sectioned in parallel with the plane to make tangential sections. Tangential sections were hybridized with an *Arc* cRNA probe and *Arc* signals from serial tangential sections were stacked and displayed on a two-dimensional plane, allowing one to easily visualize the neural activity of all pyramidal cells. We applied this method to the hippocampus of rats that had experienced contextual fear conditioning, which requires hippocampal function. We observed a net shift of *Arc* signals from dorsal to ventral CA1/subiculum with an interval prolongation to reconditioning after the initial conditioning. The reconditioning-revealed shift may reflect a reorganization process, which takes place during the period between initial conditioning and reconditioning, in the CA1/subiculum neural network that represents the neural storage and/or retrieval of the contextual fear conditioning.

Keywords: Contextual fear conditioning; Two-dimensional map; Ventral hippocampus; *Arc* gene expression; Tangential section; Neural circuit; Memory consolidation; Memory retrieval

1. Introduction

Information processing in the brain is mediated by neural networks that, upon experience, change their circuits in a plastic manner. Memory is thought to be represented on these neural networks. It is therefore very important to identify such networks and describe changes in networks during memory acquisition, consolidation, and retrieval. Generally, electrophysiological recording, histological analysis, and functional imaging are used to analyze neural networks involved in information processing during memory formation. Although these methods have a number of merits, it is quite difficult by these methods to display the entire neural activity of a given brain region such as the hippocampus, in spatially high resolution, for example, at the single cell level.

The hippocampus is involved in encoding, storage and retrieval of associations responsible for episodic memory, as well as spatial memory (O'Keefe and Dostrovsky, 1971; Morris et al., 1982; Brown and Aggleton, 2001; Eichenbaum, 2004). The hippocampus consists of four major subfields, the subiculum, CA1, CA3, and dentate gyrus. These subfields seem to be involved in distinct aspects of informational processing (Lisman, 1999). Both anatomical and lesion studies indicate that the dorsal and ventral parts of the hippocampus are functionally distinct (Maren et al., 1997; Moser and Moser, 1998; Hampson et al., 1999; Richmond et al., 1999; Kjelstrup et al., 2002; Bannerman et al., 2003). The dorsal hippocampus receives its input

* Corresponding author. Tel.: +81 427 246 318; fax: +81 427 246 318.
E-mail address: kaoru@libra.ls.m-kagaku.co.jp (K. Inokuchi).
[1] Present address: Age Dimension Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan.
[2] Present address: Division of Cerebral Structure, National Institute for Physiological Sciences, Okazaki 444-8585, Japan.

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primarily from the lateral portions of the entorhinal cortex, which receives its major inputs from various sensory and associational neocortical areas. Thus, the dorsal hippocampus is proposed to be important, specifically for the processing of exteroceptive sensory information. On the other hand, the medial portions of the entorhinal cortex, which receive an input predominantly from structures such as the amygdala, project primarily to the ventral hippocampus, suggesting that the ventral hippocampus is more important for the processing of interoceptive sensory information and emotion (Amaral and Witter, 1995). Indeed, the dorsal hippocampus plays a central role in spatial memory, whereas the ventral hippocampus is required for contextual fear conditioning (Maren et al., 1997; Moser and Moser, 1998; Richmond et al., 1999; Pittenger et al., 2002).

Here, we developed a novel method to display neural activity of the entire population of CA1/subiculum pyramidal cells by two-dimensional mapping using stacked images of tangential sections, in which the mRNA expression of one of the immediate–early genes (IEGs), *Arc*, was used as an indicator of neural activity. Among IEGs that are induced during behavioral tasks, *Arc* gene induction in the hippocampal CA1 region is specifically linked to information processing rather than general neural activities such as motor activity, environmental novelty or responses to stress (Guzowski et al., 1999, 2001; Kelly and Deadwyler, 2003). Furthermore, *Arc* mRNA expression is most sensitive to changes in behavioral tasks compared to that of the other IEGs, *c-fos* and *zif268* (Guzowski et al., 2000). Antisense oligonucleotide-mediated suppression of the Arc protein impairs long-term memory consolidation without affecting task acquisition or short-term memory (Guzowski et al., 2000). These observations make *Arc* expression suitable for the representation of a neural circuit associated with information processing. We applied this method to rats that performed contextual fear conditioning, and found a net shift of neural activity from dorsal to ventral CA1/subiculum over time after the initial conditioning. This shift may reflect a change in neural circuit associated with information coding, storage, and/or retrieval.

2. Materials and methods

2.1. Subjects and behavioral procedures

All the animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals and were approved by the Animal Care and Use Committee of the Mitsubishi Kagaku Institute of Life Science (MITILS). Naive male Wistar ST rats (8–9 weeks, 280–300 g) were housed individually in cages with free access to food and water. They were maintained in a 12 h light:12 h dark cycle, with lights on at 9:00 a.m., under constant temperature (24 ± 1 °C) in a room with a clean air conditioning system. They were handled over 1 week and then experiments were carried out in a neighboring room with the same environmental conditions.

Contextual fear conditioning was performed in a conditioning chamber that was an observation box (32 cm × 22 cm × 22 cm) made of clear and gray polyvinylchloride plates. The floor of the chamber consisted of 28 stainless steel rods (0.4 cm diameter, spaced 1 cm apart) through which foot-shocks were delivered by a scrambled-foot-shock generator SGS 002 (Muromachi Kikai Co., Tokyo, Japan). The chamber was placed in a lighted room. The chamber was cleaned with ethanol (99.5%) and dried with a hair drier for a change of air before and after the occupancy of each rat. A video camera placed in front of the chamber recorded the behavior of each rat. A controller for conditioning was operated by a remote switch placed far from the conditioning chamber.

For both conditioning and reconditioning, each rat was placed in the chamber for 5 min, and freezing behavior was observed. Then, these rats received three electric foot-shocks (0.5 mA intensity, 1.0 s duration, 30 s interval), remained in the chamber for 2 min, and then were returned to their home cage (Fig. 1A). Freezing score was counted when a rat did not move at all for the first 3 s of every 5 s interval.

2.2. Preparation of tangential sections

On the reconditioning day, all the animals were sacrificed at 30 min after the foot-shock and the hippocampus was dissected and placed in cooled phosphate-buffered saline (PBS). Whatman 3 mm paper (Maidstone, England) wetted with PBS was placed on a flat cellulose acetate filter of a vacuum filtration system (pore diameter, 0.45 μm, Corning Inc., NY, USA). Aluminum foil was extensively perforated and put on the 3 mm paper. Each dissected hippocampus was put on the flat aluminum foil with the pyramidal cell layer of the CA1 and subiculum closest to the foil. The hippocampus was covered with polyvinylidene film and the vacuum was applied, which flattened the spherical pyramidal cell layer. The flattened hippocampus was embedded in Tissue-Tek OCT compound and immediately frozen on dry ice powder (tangential block). Needle holes were punched in the tangential block as location markers and the block was then sliced in parallel with the flatten pyramidal cell layer to make serial sections (tangential sections, 10 μm thick). Approximately 20–30 serial sections containing the whole pyramidal cell layer were used for the reconstruction.

2.3. In situ hybridization

A part of the *Arc* cDNA (nucleotides 760–1380, where the adenine residue of the initiation codon is designated 1) was amplified by PCR and subcloned into the pCRII-TOPO vector (Invitrogen Corp., Carlsbad, CA, USA) to generate pCRII-situ. pCRII-situ was digested with BamHI or EcoRV

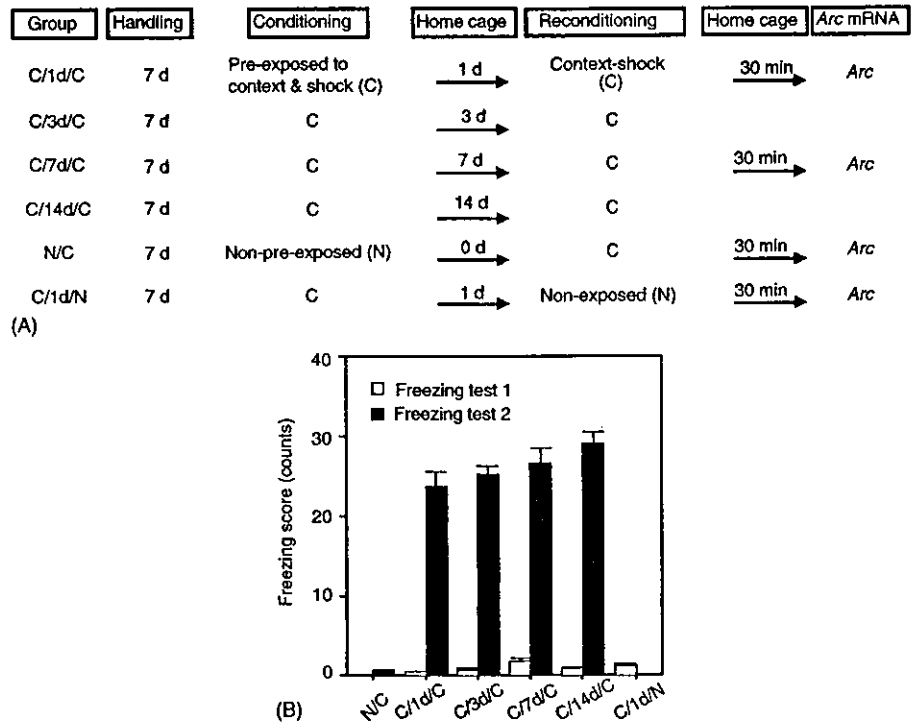

Fig. 1. Contextual fear-conditioning task. (A) Experimental design of contextual fear conditioning. (B) Freezing responses during the first 5 min of the conditioning session (freezing test 1, white bar) and during the first 5 min of reconditioning session (freezing test 2, black bar) (number of animals = 6 for each condition).

to generate templates for in vitro transcription of antisense or sense probes, respectively. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were produced by transcription with T7 and Sp6 RNA polymerases, respectively.

Tangential sections were fixed in 4% formaldehyde in PBS, pH 7.0, for 30 min, treated with 0.3% Triton X-100 in PBS, followed by incubation in 0.2N HCl for 20 min. The sections were then post-fixed with 4% formaldehyde in PBS for 10 min, and treated with 0.2% glycine in PBS for 15 min. Hybridization was performed at 42 °C, overnight, in hybridization buffer (50% formamide; 20 mM Tris–HCl, pH 8.0; 2.5 mM EDTA, pH 8.0; 300 mM NaCl; 0.25% SDS; 10% dextran sulfate; 100 mg/mL of yeast tRNA (Sigma, St. Louis, MO, USA)). Sections were washed for 1 h at 42 °C with 50% formamide in 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), and then washed twice with solution B (10 mM Tris–HCl, pH 8.0, 500 mM NaCl) at room temperature for 15 min. Sections were treated with RNase A (20 μg/mL in solution B) at 37 °C for 30 min, followed by washes with 50% formamide in 1× SSC and then with 50% formamide in 0.5× SSC for 1 h at 42 °C. After equilibration with maleate buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 1 h, sections were treated with 10% blocking reagent (Roche Diagnostics, Basel, Switzerland) in maleic acid buffer. Sections were incubated overnight at 4 °C with 1:1000 diluted alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics), washed with maleate buffer, and then equilibrated with reaction buffer (100 mM Tris–HCl, pH 9.0; 100 mM NaCl; 50 mM $MgCl_2$). Transcripts were detected with 5-bromo-4-indolyl phosphate *p*-toluidine salt and 4-nitro blue tetrazolium chloride (NBT) in the reaction buffer at 4 °C for 120 h. After recording images of the *Arc* signal, the sections were de-stained with ethanol (95%), overnight, and then re-stained with Nissl.

2.4. Reconstruction of a two-dimensional flat map

Images of *Arc* signals and Nissl-staining were acquired from sections using a 4× objective lens and a cooled CCD camera (Hamamatsu Photonics, Shizuoka, Japan) with an OptiScan Motorized Microscope Stage System (Meyer Instruments Inc., Houston, TX, USA). Acquired images were analyzed using Image Pro Express (Media Cybernetics, Silver Spring, MD, USA), Meta Morph (Universal Imaging Corporation, Downingtown, PA, USA), and Photoshop software (Adobe, San Jose, CA, USA).

Each image of a Nissl-stained serial section was handled as a layer and stacked using the Image Pro Express software. The configuration of each image was adjusted based on the positions of the marker holes (black dots in Fig. 3A) and blood vessels spanning adjacent sections. Each image of the *Arc* signal was then stacked by the same method. Background was subtracted from each *Arc* image in which the background value of each section was defined as the averaged value of four independent areas (100 × 100 pixels)

of the fiber bundle nearby CA3 (Fig. 3C, asterisk), which showed no detectable *Arc* signal.

2.5. Quantitative and statistical analyses

The *Arc* signals were binarized with appropriate threshold values so that the *Arc*-positive pixels occupied 0.5% of the total pixels in the assessed area (corresponding to the subiculum, CA1, and CA3 surrounded by the broken line in Fig. 3D) of the merged image. To display the *Arc* signal distribution in the two-dimensional map (see Fig. 4), the assessed area were divided into small areas of 20 × 20 pixels (1 pixel corresponds to 2.5 μm). The number of Arc-positive pixels was counted for each small area. Pseudo-colors, purple, blue or yellow, were assigned to each small area when it contained 1–3, 4–7 or >8 *Arc*-positive pixels, respectively. To analyze the relative *Arc* expression profile along the hippocampal longitudinal axis (see Fig. 5), the merged image was divided into 100 areas along its axis. The number of the binarized *Arc*-positive pixels in each area was counted.

Analysis of statistical significance between groups were conducted by two-way ANOVA and assessed by Fisher's PLSD.

3. Results

3.1. Behavioral paradigm

To clarify temporal changes in neural activity of the hippocampus following initial learning, we modified the paradigm of classical contextual fear conditioning (Fig. 1A). All the rats were habituated to handling for seven consecutive days. The first group of rats (C/1d/C, C/3d/C, C/7d/C, and C/14d/C) were pre-exposed to context (conditioning chamber) and shock (C) on the conditioning day. After a delay interval in their home cages of 1, 3, 7 or 14 days, the rats were again exposed to context, and freezing behavior was observed. For C/1d/C and C/7d/C, rats received an additional shock exposure immediately after the freezing test (reconditioning). In the second group, non-pre-exposed group (N/C), rats were exposed to context and freezing behavior was monitored for 5 min. The rats were then exposed to shock. The third group of rats (C/1d/N) were pre-exposed to context and shock (C), and left in their home cage for 1 day.

All the animals showed enhanced freezing behavior when they were exposed to context at various intervals after the conditioning (Fig. 1B), indicating that fear-conditioned memory was preserved over 14 days under our experimental conditions.

For *Arc* analyses, rats were sacrificed 30 min after the reconditioning session for the first two groups and after the 1 day delay in their home cage for the third group, and *Arc* mRNA expression was analyzed by in situ hybridization. Thus, all the groups of rats except the third group were exposed to context and shock 30 min prior to analyzing *Arc* mRNA expression. Differences among these animals were either an interval between conditioning and reconditioning or an experience of conditioning (Fig. 1A).

3.2. Arc mRNA expression following contextual fear conditioning

Induction of *Arc* mRNA in the hippocampal CA1 region has been linked to neural information processing (Guzowski et al., 1999; Guzowski et al., 2001; Kelly and Deadwyler, 2003). We, therefore, chose *Arc* gene expression as an indicator of neural activity that represents information processing during memory formation, consolidation, and retrieval.

Contextual fear conditioning requires the hippocampus for acquisition, storage, and retrieval of fear memory. As expected, the *Arc* mRNA signal increased in a subpopulation of neurons in the CA1 region and the subiculum 30 min after conditioning (N/C group) (Fig. 2). A similar increase in the

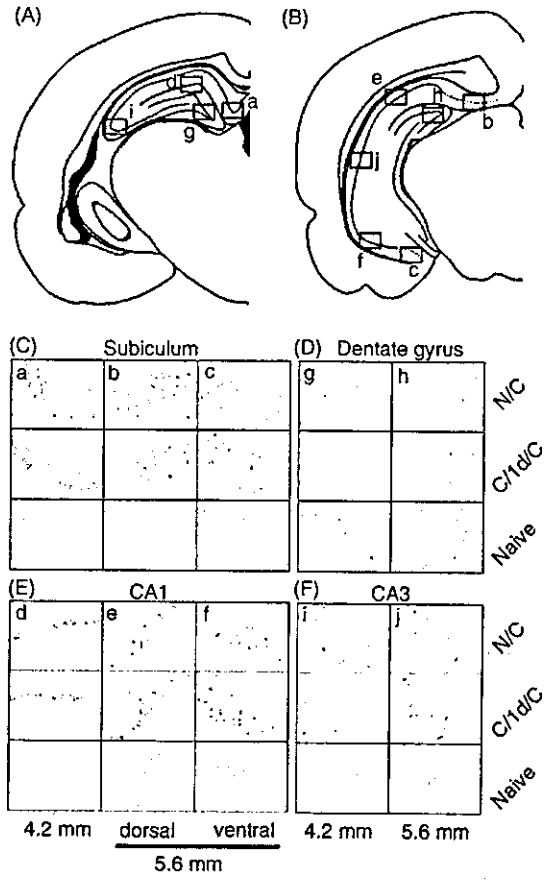

Fig. 2. Expression of *Arc* mRNA following contextual fear conditioning. (A and B) Schematic representations of coronal section at 4.2 mm (A) and 5.6 mm (B) posterior to bregma. Red squires indicate a location of pictures in (C–F). Coronal sections at 4.2 and 5.6 mm posterior to bregma were hybridized with the *Arc* cRNA probe. (C) Subiculum, (D) dentate gyrus, (E) CA1, and (F) CA3. Note that the coronal sections at 5.6 mm contain both dorsal and ventral areas of the CA1 and subiculum.

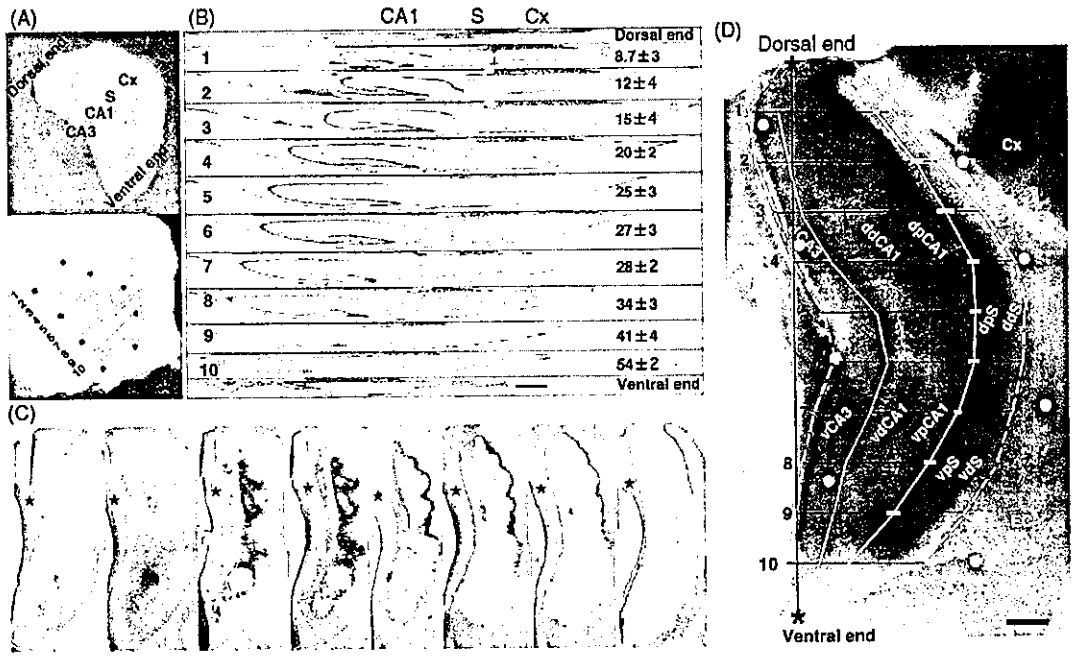

Fig. 3. Tangential sections. (A) Upper panel, dissected hippocampus containing part of the cortex. Lower panel, tangential block in which the pyramidal cell layer of the CA1 and subiculum is flattened. The black dots indicate the location of marker holes punched with a needle. Broken lines with numbers show the position of coronal sections in (B). Frozen OTC compound (white material) surrounds the tangential block. (B) Nissl-stained coronal sections (20 μm thick) from the tangential block, corresponding to the broken lines in (A). Ratio (%) of the subiculum (red line) to the CA1 (green line) + subiculum is indicated for each section (mean ± S.D., 9, tangential blocks). Note the flatness of the pyramidal cell layer. Scale bar = 1 mm. (C) Examples of the Nissl-stained tangential sections. Asterisks indicate the area used for background subtraction. (D) Two-dimensional display of the reconstructed hippocampus. A reconstructed image of the *Arc* signals obtained from C/7d/C group is shown. This image was constructed without binarization. The area including the subiculum, CA1, and CA3 is surrounded by a white broken line. The line linking the dorsal and ventral ends of the pyramidal cell layer (indicated by asterisks) was set as the longitudinal axis of the hippocampus. The border between the CA1 and subiculum was determined using the ratio of subiculum to CA1 + subiculum (see panel C), and is shown by a white line with error bars (±S.D., white horizontal bars). The border between the CA1 and CA3 is also shown by a thin white line. Black horizontal lines with numbers correspond to the position of coronal sections that are shown in panel B. White holes indicate marker holes generated by a needle. S, subiculum; dpS, dorsal–proximal subiculum; ddS, dorsal–distal subiculum; vpS, ventral–proximal subiculum; vdS, ventral–distal subiculum; dpCA1, dorsal–proximal CA1; ddCA1, dorsal–distal CA1; vpCA1, ventral–proximal CA1; vdCA1, ventral–distal CA1; dCA3, dorsal CA3; vCA3, ventral CA3; Cx, cortex; EC, entorhinal cortex. Scale bar = 1 mm.

Arc mRNA level was observed in the CA1 and subiculum of animals in the C/1d/C group (Fig. 2C and E). There seemed to be no obvious differences in the mRNA expression pattern between these two groups. *Arc* signals were not detected in the pyramidal cell layer of the naive group. In contrast, both of the N/C and C/1d/C groups showed little increase in the *Arc* signals in CA3 and dentate gyrus (Fig. 2D and F). Thus, the analysis with the coronal sections revealed that the exposure to context and shock induces *Arc* mRNA expression in the CA1 and subiculum. This analysis, however, did not examine the *Arc* expression profile in the entire hippocampus.

3.3. Preparation of sections tangential to the pyramidal cell layer

Pyramidal cells in the CA1 region and the subiculum are located at the surface of the spherical hippocampus as a thin multi-cellular layer. This topographic organization implies that the entire population of the pyramidal cells could be displayed in a two-dimensional plane. The spherical surface corresponding to the pyramidal cell layer of the dissected hippocampus was pushed against a flat plate, flattened, and frozen immediately (tangential block, Fig. 3A). When the tangential block was sliced at right angles to the flat plane (dotted lines in Fig. 3A bottom), we observed that the pyramidal cell layer of the CA1 and subiculum regions are linearly arrayed in all the sections (Fig. 3B), indicating that the pyramidal cell layer approximates a flattened plane. The frozen tangential block was then sliced in parallel with the flat plane to generate 20–30 serial sections (tangential sections) that include the entire population of the pyramidal cells in the subiculum, CA1, and CA2 regions and a part of pyramidal cells in the CA3 region.

3.4. Reconstruction of a two-dimensional map of the pyramidal cell layer

Each tangential section was hybridized with a DIG-labeled *Arc* cRNA probe and *Arc* signals were detected with the alkaline phosphatase–NBT system, followed by de-staining of the DIG signals and re-staining with Nissl.

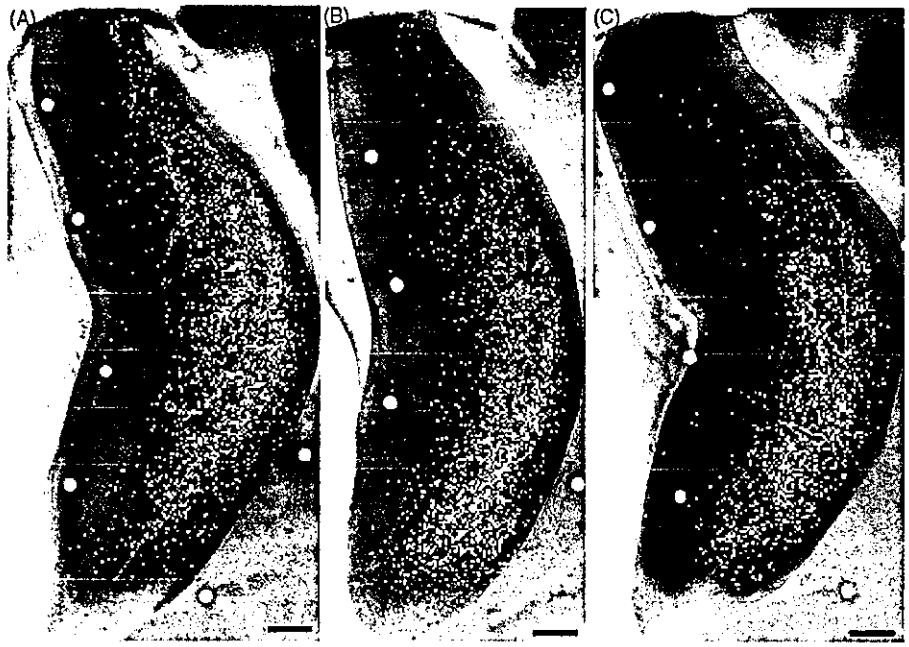

Fig. 4. *Arc* signal distribution on the two-dimensional map. Typical representations of the *Arc* signals on the hippocampus from N/C (A), C/1d/C (B), and C/7d/C (C) rats. The *Arc* signal was binarized so that the positive pixels occupied 0.5% of the total pixels in the area surrounded by broken line in Fig. 3D (see Section 2 for details). The total number of pixels within the area corresponding to the subiculum and CA1 is approximately 1.5×10^7. The red line with error bars (±S.D., horizontal bars) indicates the presumptive border between subiculum and CA1 based on the calculation in Fig. 3B. (A–C) Pseudo-coloring, purple, cyan or yellow, was assigned to each area of 20 × 20 pixels when it contained 1–3, 4–7 or >8 *Arc*-positive pixels, respectively. Scale bar = 1 mm.

Images of *Arc* and Nissl signals were obtained with a cooled CCD camera. Examples of each tangential section stained with Nissl are shown in Fig. 3C. Images of each tangential section were then stacked, using Image Pro Express software to build a two-dimensional image of the entire population of pyramidal cells. Fig. 3D shows a reconstructed map of the tangential sections hybridized with the *Arc* probe. The overall organization of the reconstructed map resembles the hippocampal flat map that has been modeled from an equally spaced series of Nissl-stained frontal sections (Swanson et al., 1978; Petrovich et al., 2001). Thus, with the technique developed here, we could easily represent the entire population of pyramidal cells in the CA1 and subiculum on a two-dimensional plane.

3.5. Distribution of the Arc mRNA signals on the two-dimensional map

Distribution of the *Arc* signal was displayed on the two-dimensional map (Fig. 4). Reconstructed images of the N/C group showed uniformly distributed *Arc* signals throughout the pyramidal cell layer with relatively dense signals along the border of the CA1 and the subiculum (Fig. 4A, see Fig. 3D for topographic assignment). In contrast, a net shift of the *Arc* signals toward the ventral area was observed in pre-exposed groups, again with higher signals at the border between the CA1 and subiculum (C/1d/C and C/7d/C) (Fig. 4B and C). Two-dimensional maps constructed from the other test animals (three animals per group) showed essentially the same *Arc* distribution patterns (data not shown).

3.6. Net shift of neural activity from the dorsal to ventral side of the pyramidal cell layer after fear conditioning

We quantified the distribution of the *Arc* signals in the pyramidal cell layer. The histogram in Fig. 5A shows the relative signal distribution along the longitudinal axis from dorsal to ventral hippocampus of individual animals. In the N/C group, the *Arc* signal distributed symmetrically along the longitudinal (dorsal–ventral) axis with a peak around the central area. The histograms for the pre-trained groups (C/1d/C and C/7d/C) did not show a symmetrical distribution pattern. An additional peak is evident at the ventral side of the hippocampus in the C/1d/C group. An overall shift of the signal toward the ventral side is also evident for the CS/7d/CS group. Cumulative analysis clearly demonstrates the net shift of *Arc* signals toward the ventral side in the pre-exposed groups (Fig. 5B) (two-way ANOVA, $F_{(2,897)} = 4.38$, $p = 0.013$; Fisher's PLSD, N/C versus C/1d/C, $p < 0.01$, N/C versus C/7d/C, $p = 0.069$). *Arc* signals were not detected in the pyramidal cell layer of the C/1d/N group (not shown), indicating that the *Arc* signals observed in the pre-exposed group were induced by the context and shock 30 min prior to the *Arc* analysis rather than the experience of the previous day.

Taken together, the previous experience of exposure to context and shock influenced the neural activation

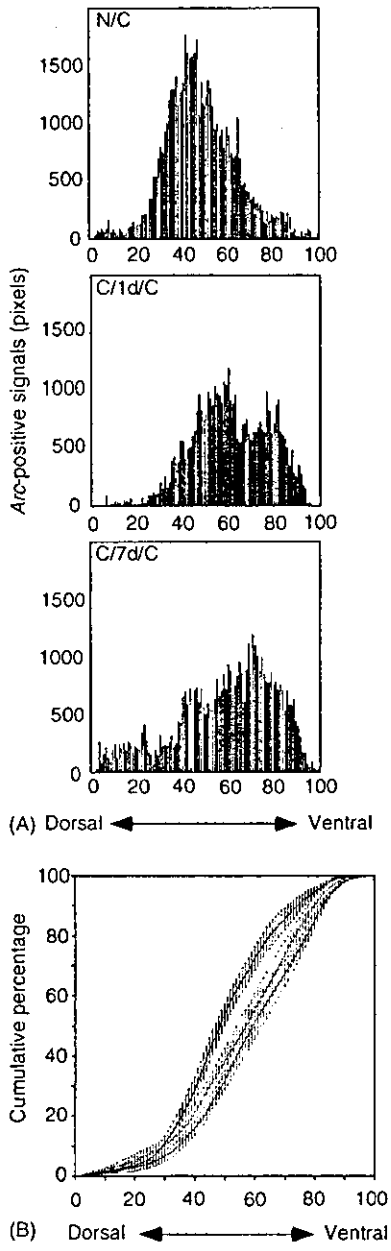

Fig. 5. Net shift of the *Arc*-positive signals from dorsal to ventral hippocampus after fear conditioning. The *Arc* signal was binarized so that the positive pixels occupied 0.5% of the total pixels in the subiculum and CA1. The area containing the subiculum and CA1 on the two-dimensional map was divided into 100 equally spaced areas along the longitudinal axis (shown in Fig. 3D) from the dorsal to ventral end. The number of *Arc*-positive pixels was counted for each area. (A) Histograms showing the distribution of the number of *Arc*-positive pixels along the longitudinal axis. (B) Cumulative percentage of the *Arc*-positive pixels. Data in panel A are displayed as a cumulative curve. Data from three animals per group were averaged (mean ± S.E.M.). Black, N/C; red, C/1d/C; green, C/7d/C.

pattern following the second exposure to context and shock. These shifts may represent a reorganization of the hippocampal neural circuit associated with the fear-conditioned memory trace.

4. Discussion

4.1. Construction of a two-dimensional map

In the present study, we developed a novel mapping method that allowed us to detect the neural activity of the entire population of pyramidal cells in the CA1/subiculum on a two-dimensional plane. All the pyramidal cells in the CA1 and subiculum were included in a total of 20–30 serial tangential sections, which easily allowed us to construct a two-dimensional flat map of the CA1 and subiculum regions. Configuration of the flat map thus constructed is quit similar to that made from a large number of coronal Nissl-stained sections (Swanson et al., 1978; Petrovich et al., 2001). Combined with the *Arc* mRNA hybridisation, the two-dimensional map technique is able to display the neural activity of the entire CA1 and subiculum regions at a spatially high resolution, even at the single cell level. Thus, the tangential section/two-dimensional map technique is a powerful method, in combination with electrophysiological recording of individual neurons in rodents and functional imaging such as functional MRI and PET in human, for the analysis of the overall pattern of neural activity in the CA1 and subiculum that is involved in information processing during memory acquisition, consolidation, retrieval, and reconsolidation.

4.2. Role of the ventral hippocampus revealed by analysis of the two-dimensional map

The most pronounced feature of the *Arc* signal distribution in the two-dimensional map is the net shift of *Arc*-positive cells from dorsal to ventral CA1/subiculum in the pre-exposed group (C/1d/C and C/7d/C). Both groups of rats, non-pre-exposed and pre-exposed, received the same input 30 min prior to sacrifice on the last day. Thus, the shift can be attributed to the experience of pre-exposure to context and shock, 1 or 7 days prior to shock on the reconditioning day. In the N/C group, the animals were exposed to the conditioned box as a novel environment 30 min prior to the *Arc* analysis. These animals showed a searching behavior and acquired the surrounding spatial information until they received electric foot-shocks. In contrast, animals of the pre-exposed groups showed a freezing behavior at the beginning of the reconditioning session, as they had already learned the association between context and shock. The difference in the distribution of the *Arc*-positive cells, therefore, reflects the different performance of these two groups of animals, and may be caused by the difference in the information processing between encoding/consolidation in the N/C group and retrieval/re-encoding/reconsolidation in the pre-exposed group.

Contextual fear conditioning requires the hippocampus, specifically the ventral hippocampus, and the amygdala (Kim and Fanselow, 1992; Kim and Davis, 1993; Maren et al., 1997; Fanselow, 2000; Pare et al., 2004). The amygdala is

implicated in emotion, memory and social behavior, and plays a critical role in fear memory retrieval (LeDoux, 2000; Rodrigues et al., 2004). Afferent and efferent fibers from the amygdala project mainly to the ventral hippocampus, in particular, to the ventral–proximal CA1 and ventral–proximal subiculum (Naber and Witter, 1998; Petrovich et al., 2001), precisely where the most intense *Arc* signals were detected in the pre-exposed group. The ventral–proximal subiculum is the major origin of output from the hippocampus to the amygdala and anterior hypothalamic nucleus, which both play a central role in the expression of aggressive–defensive behavior (Naber and Witter, 1998; Kishi et al., 2000). Therefore, the relative increase in neural activity in the ventral–proximal CA1/subiculum may reflect the reinforcement of the amygdala and ventral–proximal CA1/subiculum circuit by repeated fear experiences, which induce the fear memory retrieval process. Consistent with this model, imaging studies in human subjects reveal that the encoding and retrieval of memories appears to involve the anterior (corresponding to dorsal in rodents) and posterior (ventral in rodents) hippocampus, respectively (Lepage et al., 1998; Zeineh et al., 2003). The relative increase in neural activity in the ventral–proximal CA1/subiculum probably reflects the contribution of this area to the retrieval of fear memory. This does not, however, necessarily exclude the possibility that reconditioning itself may affect the *Arc* expression profile.

Previously consolidated fear memories enter a labile and unstable state when recalled. Memories then enter a new phase referred to as the reconsolidation process, in order to be reinforced and maintained (Misanin et al., 1968; Mactutus et al., 1979; Nader et al., 2000). In this context, the net shift of the *Arc* signals from dorsal to ventral hippocampus in the pre-exposed groups may also reflect an involvement of the ventral hippocampus in reconsolidation after retrieval. Thus, our results suggest that the reconsolidation of fear memory uses specific neural pathways that are distinct from those utilized during the consolidation of a new memory.

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References

Amaral, D.G., Witter, M.P., 1995. Hippocampal formation. In: Paxinos, G. (Ed.), The Rat Nervous System. Academic Press, San Diego, pp. 443–493.

Bannerman, D.M., Grubb, M., Deacon, R.M., Yee, B.K., Feldon, J., Rawlins, J.N., 2003. Ventral hippocampal lesions affect anxiety but not spatial learning. Behav. Brain Res. 139, 197–213.

Brown, M.W., Aggleton, J.P., 2001. Recognition memory: what are the roles of the perirhinal cortex and hippocampus? Nat. Rev. Neurosci. 2, 51–61.

Eichenbaum, H., 2004. Hippocampus: cognitive processes and neural representations that underlie declarative memory. Neuron 44, 109–120.

Fanselow, M.S., 2000. Contextual fear, gestalt memories, and the hippocampus. Behav. Brain Res. 110, 73–81.

Guzowski, J.F., Lyford, G.L., Stevenson, G.D., Houston, F.P., McGaugh, J.L., Worley, P.F., Barnes, C.A., 2000. Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. J. Neurosci. 20, 3993–4001.

Guzowski, J.F., McNaughton, B.L., Barnes, C.A., Worley, P.F., 1999. Environment-specific expression of the immediate-early gene *Arc* in hippocampal neuronal ensembles. Nat. Neurosci. 2, 1120–1124.

Guzowski, J.F., Setlow, B., Wagner, E.K., McGaugh, J.L., 2001. Experience-dependent gene expression in the rat hippocampus after spatial learning: a comparison of the immediate-early genes *Arc*, c-*fos*, and *zif268*. J. Neurosci. 21, 5089–5098.

Hampson, R.E., Simeral, J.D., Deadwyler, S.A., 1999. Distribution of spatial and nonspatial information in dorsal hippocampus. Nature 402, 610–614.

Kelly, M.P., Deadwyler, S.A., 2003. Experience-dependent regulation of the immediate-early gene *Arc* differs across brain regions. J. Neurosci. 23, 6443–6451.

Kim, J.J., Fanselow, M.S., 1992. Modality-specific retrograde amnesia of fear. Science 256, 675–677.

Kim, M., Davis, M., 1993. Lack of a temporal gradient of retrograde amnesia in rats with amygdala lesions assessed with the fear-potentiated startle paradigm. Behav. Neurosci. 107, 1088–1092.

Kishi, T., Tsumori, T., Ono, K., Yokota, S., Ishino, H., Yasui, Y., 2000. Topographical organization of projections from the subiculum to the hypothalamus in the rat. J. Comp. Neurol. 419, 205–222.

Kjelstrup, K.G., Tuvnes, F.A., Steffenach, H.A., Murison, R., Moser, E.I., Moser, M.B., 2002. Reduced fear expression after lesions of the ventral hippocampus. Proc. Natl. Acad. Sci. U.S.A. 99, 10825–10830.

LeDoux, J.E., 2000. Emotion circuits in the brain. Annu. Rev. Neurosci. 23, 155–184.

Lepage, M., Habib, R., Tulving, E., 1998. Hippocampal PET activations of memory encoding and retrieval: the HIPER model. Hippocampus 8, 313–322.

Lisman, J.E., 1999. Relating hippocampal circuitry to function: recall of memory sequences by reciprocal dentate–CA3 interactions. Neuron 22, 233–242.

Mactutus, C.F., Riccio, D.C., Ferek, J.M., 1979. Retrograde amnesia for old (reactivated) memory: some anomalous characteristics. Science 204, 1319–1320.

Maren, S., Aharonov, G., Fanselow, M.S., 1997. Neurotoxic lesions of the dorsal hippocampus and Pavlovian fear conditioning in rats. Behav. Brain Res. 88, 261–274.

Misanin, J.R., Miller, R.R., Lewis, D.J., 1968. Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. Science 160, 554–555.

Morris, R.G.M., Garrud, P., Rawlins, J.N.P., O'Keefe, J., 1982. Place navigation impaired in rats with hippocampal lesions. Nature 297, 681–683.

Moser, M.B., Moser, E.I., 1998. Functional differentiation in the hippocampus. Hippocampus 8, 608–619.

Naber, P.A., Witter, M.P., 1998. Subicular efferents are organized mostly as parallel projections: a double-labeling, retrograde-tracing study in the rat. J. Comp. Neurol. 393, 284–297.