Table 1: Demographic characteristics of patients with schizophrenia and controls

			Control Group	Patient Group	Statistical value (*df*)	*P* value
n			49	27		
Age (years)						
Mean			37.4 ± 12.3	44.3 ± 12.7	t (74) = 2.1	0.039
Range			22–72	26–72		
Males/females			14/35	16/11	χ^2 (1) = 6.9	0.009
Handedness scale						
Right/Left			46/3	26/1		
Education level (years)						
Mean			16.6 ± 3.4	13.3 ± 2.4	t (73) = 4.4	0.00004
Range			12–24	9–18		
Family history +/–[a]				7/20		
Outpatient/inpatient				12/15		
Age of onset (years)						
Mean				23.4 ± 7.7		
Range				13–42		
Antipsychotic medication[b]						
Single dose	Typical	n = 7		782.2 ± 631.4		
	Atypical	n = 7		421.4 ± 196.1		
Dual dose	Typical	n = 13		529.1 ± 721.0		
	Atypical			702.6 ± 606.4		
Number of hospitalizations						
Mean			–	2.9 ± 2.7		
Range			–	0–11		
Hospitalized duration, months						
Mean			–	82.5 ± 150.8		
Range			–	0–624		

[a] Positive family history was defined as at least one schizophrenic individual within the second degree relatives.

[b] Chlorpromazine equivalent (mg/day).

Table 2: Comparisons in motor and cognitive tasks between patients with schizophrenia and controls

	Control group	Patient group	F [a]	P
A. Motor tasks				
Pegboard	67.0 ±11.6	132.3 ±65.6	19.9	3.0×10^{-5}
Normal Drawing	15.5 ±4.0	27.3 ±9.7	22.9	9.1×10^{-6}
Finger Movement	21.7 ±3.1	13.0 ±12.9	30.7	5.0×10^{-7}
B. Cognitive tasks				
WMS-R				
Verbal Memory	110.2 ±12.9	76.6 ±19.5	31.8	3.3×10^{-7}
Visual Memory	109.1 ±11.9	73.4 ±19.0	42.0	1.1×10^{-8}
General Memory	110.8 ±12.0	72.1 ±19.2	48.1	1.6×10^{-9}
Attention & Concentration	107.3 ±13.9	83.7 ±15.0	28.3	1.2×10^{-6}
Delayed Recall	112.0 ±10.1	69.2 ±18.5	79.0	4.3×10^{-13}
WAIS-R				
Verbal IQ	109.9 ±13.2	85.0 ±19.7	23.9	6.1×10^{-6}
Performance IQ	108.2 ±11.1	76.2 ±16.8	41.1	1.4×10^{-8}
Full scale IQ	109.9 ±11.4	79.3 ±18.9	40.6	1.7×10^{-8}
WCST [b]				
Categories Completed	4.1 ±1.98	2.1 ±2.17	4.0	0.051

[a] ANCOVA controlling for age, sex, and education years; $df_1 = 1$, $df_2 = 70$
[b] ANCOVA controlling for age, sex, and education years; $df_1 = 1$, $df_2 = 64$

Table 3: Comparisons in motor and cognitive tasks between patients with schizophrenia and controls matched for age and education level

	Controls	Patients	*df*	*t* value	*P* value
A Motor Tests					
Pegboard	67.8 ± 13.2	99.5 ± 31.3	16.1	3.4	< 0.01
Normal Drawing	16.9 ± 4.2	22.3 ± 7.7	18.7	2.2	< 0.05
Finger Movement	21.2 ± 3.1	14.8 ± 7.5	16.0	2.8	< 0.05
B Cognitive Tests					
WAIS-R					
Verbal Memory	107.8 ± 10.0	82.5 ± 21.9	16.9	3.8	< 0.01
Visual Memory	104.7 ± 15.4	80.6 ± 22.7	24.0	3.2	< 0.01
General Memory	107.8 ± 11.6	79.9 ± 21.0	24.0	4.2	< 0.001
Attention & Concentration	106.0 ± 13.6	83.1 ± 14.5	24.0	4.2	< 0.001
Delayed Recall	110.8 ± 10.4	74.0 ± 20.5	17.8	5.8	< 0.0001
WMS-R					
Verbal IQ	109.2 ± 12.6	90.2 ± 16.8	24.0	3.2	< 0.01
Performance IQ	105.9 ± 13.7	81.2 ± 16.2	24.0	4.2	< 0.001
Full Scale IQ	108.3 ± 12.2	84.9 ± 15.1	24.0	4.3	< 0.001
WCST					
Categories Completed	3.6 ± 2.3	2.5 ± 2.4	22.0	1.1	0.268

Table 4: Correlation of demographic and clinical characteristics with delayed recall and finger movement

	Delayed recall		Finger movement	
	r	*P*	*r*	*P*
Age	−0.05	0.81	−0.36	0.06
Education level (years)	0.51	0.01	0.05	0.80
Age of onset	0.17	0.39	0.03	0.90
Duration of illness	−0.18	0.38	−0.37	0.06
Antipsychotic medication [a]	−0.33	0.09	−0.04	0.84
Number of hospitalizations	−0.29	0.15	−0.25	0.20
Hospitalized duration	−0.00	1.00	−0.35	0.07

[a] Equivalent to chlorpromazine.

r = Pearson's product moment correlation coefficient.

Table 5: Relationship of categorical variables with delayed recall and finger movement

Categories (*n*)	Delayed Recall				Finger Movement			
	Mean	SD	*t* value [a]	*P* value	Mean	SD	*t* value [a]	*P* value
Male (16)	70.3	18.8	0.4	0.719	13.5	6.2	0.3	0.735
Female (11)	67.6	18.8			12.5	8.7		
Outpatient (12)	70.0	17.3	0.2	0.849	17.0	6.9	2.6	0.014
Inpatient (15)	68.6	19.9			9.	7.0		
Familial history + (7)	61.6	15.5	1.3	0.209	14.6	6.6	–0.6	0.559
Familial history – (20)	71.9	19.0			12.6	8.1		

[a] *t*-test for independent groups; $df = 2$

Table 6: Correlation of motor and cognitive functions with delayed recall and finger movement

	Delayed Recall		Finger Movement	
	r	*P*	*r*	*P*
Pegboard	− 0.27	0.18	− 0.51	0.01
Normal Drawing	− 0.44	0.02	− 0.32	0.10
Finger Movement	− 0.00	1.00	–	–
General Memory (WMS-R)	0.88	0.00	0.73	0.72
Attention and Concentration (WMS-R)	0.45	0.02	0.20	0.32
Delayed Recall (WMS-R)	–	–	− 0.00	1.00
Full Scale IQ (WAIS-R)	0.65	0.00	0.21	0.30
Completed Categories (WCST)	0.34	0.12	0.34	0.11

r = Pearson's product moment correlation coefficient

Figure Captions

Figure 1: An example of a drawing test result

This figure is a sample stimulus used in the normal drawing test and mirror drawing test. The figure was 10 cm in diameter and 3 mm in width. This upper, interrupted drawing was one patient's typical result of mirror drawing. He could not continue the drawing and gave up 2 min after starting the test.

Figure 2: Finger movement test

The subjects were asked to rotate each finger clockwise and counterclockwise with the remaining fingers extended and fixed. The figure presents the third-finger condition.

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Brief report

IQ decline and memory impairment in Japanese patients with chronic schizophrenia

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Abstract

The extent of IQ decline due to the development of illness in patients with chronic schizophrenia and the degree of memory impairment relative to such IQ decline still remain unclear. Our results suggest that schizophrenia patients experience marked IQ decline due to the development of illness and their wide-ranging memory impairments are even more severe than the IQ decline.

Keywords: Schizophrenia; IQ; Memory

1. Introduction

Cognitive impairment is a core feature of schizophrenia, with a great impact on patients' daily lives. Those therapies that have the potential to improve cognitive deficits of patients with schizophrenia, including cognitive remediation therapy (Medalia et al., 1998; Wykes et al., 2003), as well as the favorable effects of atypical antipsychotic drugs on cognition (Bilder et al., 2002; Harvey et al., 2006; Keefe et al., 2006), have been attracting increasing attention from researchers and clinicians. From this viewpoint, the precise delineation of cognitive impairments in schizophrenia patients is essential.

Intellectual deficits in patients with chronic schizophrenia have been reliably identified (Heinrichs and Zakzanis, 1998; Dickinson et al., 2004) with some ongoing debate as to "whether it is possible to be schizophrenic yet neuropsychologically normal" (Palmer et al., 1997; Kremen et al., 2000; Wilk et al., 2005); however, the extent of IQ decline caused by the development of schizophrenia remains unclear because the premorbid IQ scores of persons who later develop schizophrenia are lower than

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those of their peers (Fuller et al., 2002; Reichenberg et al., 2005). Impairments in memory, working memory, and attention in patients with schizophrenia are well documented (Aleman et al., 1999; Silver et al., 2003; Hori et al., 2006), but the relationship of these cognitive deficits to the possible decline in IQ has not been established. Here we assessed cognitive functions including intellectual and wide-ranging memory functioning in patients with chronic schizophrenia in relation to age- and premorbid IQ-matched healthy controls.

2. Materials and methods

Eighty-two patients who met the DSM-IV criteria (American Psychiatric Association, 1994) for schizophrenia participated in this study. All patients were receiving antipsychotic drugs at the National Center of Neurology and Psychiatry (NCNP), Musashi Hospital and were clinically stable at the time of the neuropsychological tests. Eighty-two age- and premorbid IQ-matched healthy volunteers were recruited from hospital staff and their associates and also from the community. Healthy participants were interviewed by a research psychiatrist using the Japanese version of the Mini-International Neuropsychiatric Interview (MINI, Sheehan et al., 1998) to confirm the absence of any psychiatric illnesses. A portion of the subjects were from our previous sample (Hori et al., 2006). Written informed consent was obtained from all subjects prior to their inclusion in the study. The study was approved by the ethics committee of the NCNP.

Premorbid IQ was estimated with the Japanese Adult Reading Test (JART, Matsuoka et al., 2002; 2006), a Japanese version of the National Adult Reading Test (NART, Nelson and Wilson, 1991). This test is considered to provide an estimate of premorbid IQ in schizophrenia patients (Uetsuki et al., 2006), which is consistent with the original NART (Crawford et al., 1992; O'Carroll et al., 1992). In this test, subjects were required to read out 100 idioms of Han-Chinese characters (Japanese kanji characters). JART-estimated premorbid IQ was calculated for each subject according to previous reports (Matsuoka et al., 2002, 2006). The full version of the Wechsler Memory Scale-Revised (WMS-R, Wechsler, 1987; Sugishita, 2001) was administered to all participants. Outcome measures of the WMS-R were verbal memory, visual memory, delayed recall, auditory attention, visual attention, verbal working memory, and visual working memory. To precisely assess subjects' current intellectual function, a full version of the Wechsler Adult Intelligence Scale-Revised (WAIS-R, Wechsler, 1981; Shinagawa et al., 1990) was administered, yielding age-corrected indices of verbal, performance, and full-scale IQs.

Schizophrenic symptoms were assessed by an experienced research psychiatrist in 46 of the 82 patients using the Positive and Negative Syndrome Scale (PANSS, Kay et al., 1987). Daily doses of antipsychotics and anticholinergic antiparkinsonian drugs were converted to chlorpromazine equivalents (CPZeq) and biperiden equivalents (BPDeq), respectively, using published guidelines (American Psychiatric Association, 1997; Inagaki et al., 1999; Minzenberg et al., 2004).

Results are reported as mean ± standard deviation (S.D.). Demographic characteristics and cognitive test results were compared between groups. We used *t*-test or analysis of variance (ANOVA) to compare mean scores and the χ^2 tests to compare categorical variables. Analysis of covariance (ANCOVA) was used to compare means between groups, controlling for confounding variables. Statistical significance was set at two-tailed $P<0.05$. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 11.0 (SPSS Japan, Tokyo).

3. Results

Male/female ratios of patients and controls were 48/34 and 25/57, respectively, indicating that the patient group had a greater representation of males ($\chi^2(1)=13.06$, $P<0.001$). The mean ages of the patients and controls were 44.3±13.8 and 44.2±14.9, respectively ($t=0.05$, $df=162$, $P=0.96$). The mean years of education of the patients and controls were 13.4±2.5 and 14.1±2.2, respectively ($t=1.88$, $df=162$, $P=0.06$). The JART-predicted premorbid IQ scores of patients and controls were 102.2±11.6 and 102.3±7.4, respectively ($t=0.46$, $df=137.8$, $P=0.96$). Of the 82 patients, 56 were outpatients and 26 were inpatients. The mean age of illness onset was 24.7±8.8. Illness duration was 19.6±13.7 years, demonstrating that our patients were in the chronic phase of schizophrenia. CPZeq and BPDeq were 781.7±710.1 and 2.2±2.0, respectively. PANSS positive, negative, and total scores were 13.9±6.7, 19.1±7.1, and 62.1±17.9, respectively.

Verbal, performance, and full-scale IQs of patients with schizophrenia and healthy controls are presented in Supplementary Table 1. ANOVA showed that these three IQ indices in patients were significantly lower than those in controls (all $P<0.001$). The VIQ/PIQ ratios of patients and controls were 1.08±0.18 and 0.95±0.11, respectively ($F=22.5$, $df=1,160$, $P<0.001$, by ANCOVA with gender as a covariate). Scores of 13 subscales of the WMS-R in patients and controls are also shown in Supplementary Table 1. Patients performed significantly

more poorly than controls on all these cognitive domains (all $P<0.001$), except for auditory attention ($P=0.15$). Fig. 1(a) shows mean scores of the patients and controls on JART-estimated IQ, WAIS-R full-scale IQ, and the main three memory indices of the WMS-R. Dips of current IQ and all memory domains in patients are apparent, although the two groups are matched for the JART-estimated premorbid IQ.

To control for the current IQ and gender effects on these test results, ANCOVA was used with full-scale IQ and gender as covariates. It revealed that patients performed significantly more poorly than controls on verbal memory, visual memory, delayed recall, visual attention, and verbal working memory, even after controlling for full-scale IQ and gender (Supplementary Table 1). To confirm these results, additional comparisons were made between patients whose current IQ scores were within normal limit (IQ-WNL patients, defined as WAIS-R full-scale IQ ≥ equal to or greater than 85; $n=46$) and total controls ($n=82$). Fig. 1(b) summarizes the results, showing that there was no difference in current IQ between IQ-WNL patients (mean IQ: 98.85±8.55) and controls (mean IQ: 101.95±11.30), while these patients still showed significantly lower scores on all three memory indices compared with controls. On the other hand, the JART-estimated premorbid IQ of IQ-WNL patients was significantly higher than that of controls.

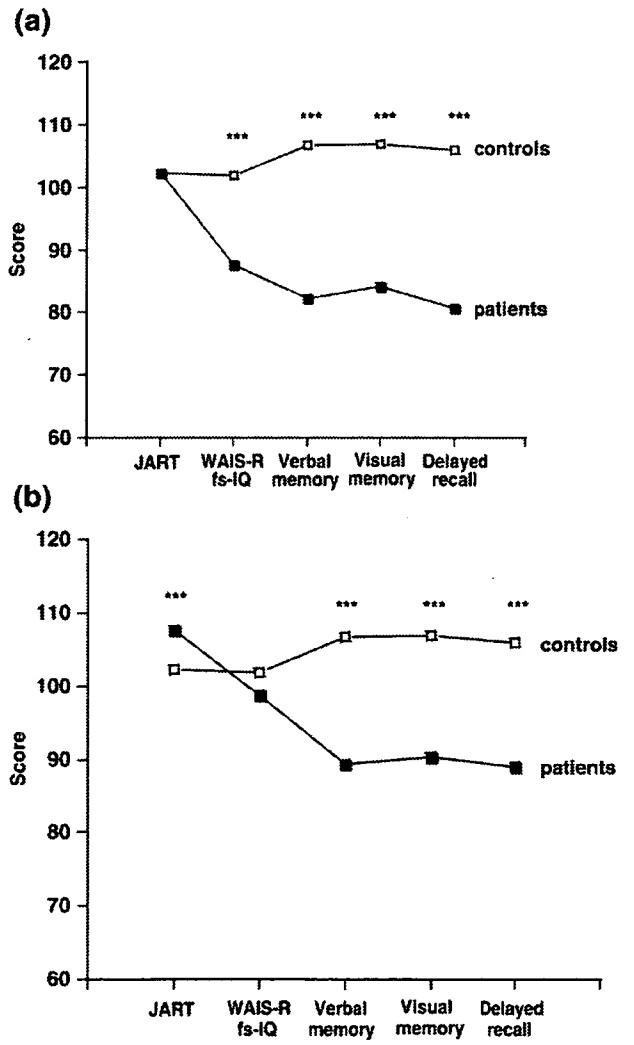

Fig. 1. Mean scores of patients and controls on JART IQ, WAIS-R full-scale IQ, Verbal memory, Visual memory, and Delayed recall indices (WMS-R). (a) total patients ($n=82$) vs. total controls ($n=82$) and (b) IQ-WNL patients (defined as WAIS-R full-scale IQ ≥ 85, $n=46$) vs. total controls ($n=82$).***$P<0.001$.

4. Discussion

In the present study we examined intellectual and memory functions in patients with chronic schizophrenia relative to age- and premorbid IQ-matched healthy controls. Our results confirmed that patients with chronic schizophrenia have wide-ranging cognitive impairments, consistent with the literature on schizophrenia.

The relationship of the development of schizophrenia to declining IQ scores has been confounded by findings that premorbid intelligence itself is likely to be lower in persons who later develop schizophrenia than in their peers (Fuller et al., 2002; Reichenberg et al., 2005). To address this issue, we employed a premorbid IQ-matched case-control sample. Although the cross-sectional nature of the present study does not allow any definite conclusions to be drawn concerning the time when the IQ decline actually occurred (i.e., during the prodromal stage, immediately after illness onset, or during the chronic course of illness), the observed differences in current IQs between patients and controls provide evidence for marked IQ decline due to the development of schizophrenia. Means of estimated premorbid IQ and current full-scale IQ in patients were 102.20 and 87.68, respectively, suggesting an approximate 1 S.D. decline in IQ score related to the development of illness. On the other hand, the subgroup of patients whose current IQ was within normal limits (and thus similar to that of controls) showed significantly higher premorbid IQ as estimated by the JART than controls (Fig. 1(b)), which favors the view that even neuropsychologically normal patients with chronic schizophrenia have compromised cognitive functioning relative to their presumed premorbid level of intellectual function (Kremen et al., 2000). Furthermore, in the present study performance IQ of the patients was more severely impaired than verbal IQ, congruent with prior reports (Heinrichs and Zakzanis, 1998).

Pervasive memory impairment in patients with schizophrenia relative to premorbid IQ-matched controls was

found, and most deficits remained significant even after current IQ was controlled for, supporting that memory impairment is a core feature of schizophrenia (Saykin et al., 1991; Heinrichs and Zakzanis, 1998; Aleman et al., 1999). The marked impairment in verbal memory is consistent with numerous studies (e.g., Saykin et al., 1991; Heinrichs and Zakzanis, 1998). Although visual memory deficits in schizophrenia have attracted less attention from researchers than verbal memory, several studies have reported substantial impairment of visual memory (Saykin et al., 1991; Aleman et al., 1999), consistent with the present study. The pronounced impairment in delayed recall observed here is also in line with prior reports (Aleman et al., 1999; Dickinson et al., 2004). Deficits of verbal and spatial working memory in schizophrenia tapped by the Wechsler digit span backward and spatial span backward subtests, respectively, are fairly consistent findings (Conklin et al., 2000; Silver et al., 2003; Dickinson et al., 2004), which were replicated in the current study. Previous studies have reported that the performance on the forward digit span task of schizophrenia patients is significantly poorer than that of healthy people, indicating impaired attentional function in schizophrenia (Conklin et al., 2000; Silver et al., 2003). The findings of the present study, by contrast, suggest that auditory attention as measured by the forward digit span subtest is preserved in schizophrenia. The discrepant findings regarding auditory attention in the present study relative to previous ones might be due in part to the distinct matching status between patients and controls regarding education and premorbid IQ.

In conclusion, our results suggest that patients with chronic schizophrenia have substantially lower intellectual function relative to their presumed premorbid level and that their memory impairment is even more severe than the IQ decline. To definitively delineate the lifetime course of cognitive decline in schizophrenia, longitudinal studies that range from childhood to the chronic phase are needed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2007.11.002.

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A possible association between missense polymorphism of the breakpoint cluster region gene and lithium prophylaxis in bipolar disorder

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Abstract

Lithium is one of the most commonly used drugs for the treatment of bipolar disorder. To prescribe lithium appropriately to patients, predictors of response to this drug were explored, and several genetic markers are considered to be good candidates. We previously reported a significant association between genetic variations in the breakpoint cluster region (BCR) gene and bipolar disorder. In this study, we examined a possible relationship between response to maintenance treatment of lithium and Asn796Ser single-nucleotide polymorphism in the BCR gene. Genotyping was performed in 161 bipolar patients who had been taking lithium for at least 1 year, and they were classified into responders for lithium monotherapy and non-responders. We found that the allele frequency of Ser796 was significantly higher in non-responders than in responders. Further investigation is warranted to confirm our findings.

Keywords: BCR (breakpoint cluster region); Bipolar disorder; Lithium; SNP (single-nucleotide polymorphism)

1. Introduction

Bipolar disorder (BPD) is one of the most distinct syndromes in psychiatry, which is characterized by recurrent episodes of mania and depression. Three representative mood stabilizers, lithium, valproate and carbamazepine, are used worldwide for its treatment, and American Psychiatric Association guideline listed lithium as a first line agent (American Psychiatric Association, 2002). However, these treatments are associated with variable rates of efficacy and often with intolerable side effects. Therefore, many researchers explored psychopathological and biological markers for good response to lithium treatment (Gelenberg and Pies, 2003; Ikeda and Kato, 2003). To date, several studies investigated possible molecular predictors of lithium efficacy. The functional polymorphism in the upstream regulatory region of the serotonin transporter gene (5-HTTLPR) has been associated with lithium efficacy in two independent studies (Serretti et al., 2001;

Abbreviations: ANOVA, analysis of variance; BCR, breakpoint cluster region; BDNF, brain-derived neurotrophic factor; BPD, bipolar disorder; BP I, bipolar I disorder; BP II, bipolar II disorder; PH domain, pleckstrin homology domain; SNP, single-nucleotide polymorphism.

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Del Zompo et al., 1999), although the polymorphism associated with better lithium response was opposite. Other numerous genetic variants including catechol-*O*-methyltransferase were not associated with lithium response (Serretti et al., 2002). The association between prophylactic lithium response and the polymorphism of the brain-derived neurotrophic factor (BDNF) gene was reported (Rybakowski et al., 2005); however, this association was not replicated in subsequent studies (Masui et al., 2006; Michelon et al., 2006).

We previously reported a significant association between genetic variants in the breakpoint cluster region gene (*BCR*), which is located on chromosome 22q11, and BPD (Hashimoto et al., 2005). The *BCR* is highly expressed in hippocampal pyramidal cell layer and dentate gyrus (Fioretos et al., 1995), and encodes a Rho GTPase-activating protein (GAP), which inactivate the Rho GTPase playing an important role in neuronal development (Diekmann et al., 1991; Negishi and Katoh, 2002). The A2387G single-nucleotide polymorphism (SNP) in the *BCR* gene [National Center for Biotechnology Information (NCBI) SNP ID: rs140504] is the non-conservative SNP giving rise to an amino acid change of asparagine to serine at codon 796 (Asn796Ser; NCBI Protein ID: NP_004318). Ser796 allele showed a significant association with BPD and stronger evidence for an association with bipolar II disorder (BPII) than bipolar I disorder (BPI) (Hashimoto et al., 2005). It has been reported that patients with BPII have greater number of abnormal mood episodes and comorbidity of other psychiatric illnesses than patients with BPI (Ayuso-Gutierrez and Ramos-Brieva, 1982; Berk and Dodd, 2005). These clinical features of BPII have been also considered as markers for poor response to lithium treatment (Ikeda and Kato, 2003). Therefore, Ser796 allele of the *BCR* gene may contribute to poorer response to lithium therapy in BPD.

In this study, we examined the possible association between prophylactic effect of lithium and Asn796Ser SNP of the *BCR* gene in Japanese patients with BPD.

2. Methods

2.1. Subjects

Subjects were 161 patients with BPD (83 patients were BPI, and 78 patients were BPII). Consensus diagnosis was made for each patient by at least two psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV) criteria. The presence of concomitant diagnoses of mental retardation, drug dependence, or other Axis I disorder, together with somatic or neurological illnesses that impaired psychiatric evaluation, represented exclusion criteria. They were composed of 76 males and 85 females with mean age of 48.2± 12.8 years (mean±S.D.). All the subjects were biologically unrelated Japanese. Patients had been treated with lithium carbonate and its serum concentration was maintained between 0.4 and 1.2 mEq/L at least for one year, in a completely naturalistic setting.

Response to lithium treatment was retrospectively determined for each patient from all available information including clinical interview and medical records, by at least two psychiatrists, and the patients were classified into lithium responders and non-responders. The phenotype definition of lithium prophylaxis is a very difficult issue. Lithium responders were defined as those patients without any affective episodes during the maintenance period of lithium mono-therapy. During the maintenance period, the addition of antidepressants, antipsychotics, or anticonvulsants was regarded as a relapse, and excluded from the responder group. However, coadministration of hypnotics for sleep disturbance was allowed, and was not regarded as a relapse when subsequent affective episode did not appear.

Our definition of response to lithium treatment is full response without any affective episode during lithium treatment. This definition is similar to "excellent lithium responders" used as clinical endophenotypic marker of BPD in some molecular-genetic research (Rybakowski et al., 2005; Mamdani et al., 2007). On the other hand, recurrence index [number of episodes/duration of illness (years)] before and during lithium treatment is a better method to measure the response to lithium including partial response (Gasperini et al., 1993; Serretti et al., 2002). However, more clinical information is necessary to calculate the recurrence index. We investigated the association between the change of recurrence index and clinical variables in parts of total subjects (24 patients) whose recurrence pattern were clearly established during more than 1 year [mean 5.8±5.0 (range 1.3–21.0) years] before lithium treatment. They were composed of 9 BPI and 15 BPII patients, whose age of onset was 35.4± 9.5 years old, duration from onset of illness to lithium treatment was 9.5±7.0 (range 1.3–22.0) years, number of episodes which could be clearly identified before lithium treatment was 16.3± 30.3 (range 3.0–150.0), duration of lithium treatment was 6.0± 4.3 (range 1.0–14.3) years, number of episodes during lithium treatment was 6.8±6.0 (range 0.0–26.0) and recurrence index before and during lithium were 2.7±2.8 (range 0.6–14.2) and 1.8±1.5 (range 0.0–5.3), respectively.

After complete description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethics committees.

Table 1
Clinical characteristics of subjects, sorted by response to lithium treatment

	Response to lithium treatment		
	Responders (N=43)	Non-responders (N=118)	
Subtype			χ^2test
BPI	29 (34.9%)	54 (65.1%)	$p<0.05$
BPII	14 (18.0%)	64 (82.0%)	
Gender			
Male	25 (32.9%)	51 (67.1%)	NS
Female	18 (21.2%)	67 (78.8%)	
			t-test
Age at last observation	54.4±11.8	46.1±12.4	$p<0.01$
Age of onset	41.5±13.6	32.9±10.7	$p<0.01$
Duration of illness	12.9±9.0	13.2±9.9	NS

Continuous values were represented as the mean±SD.
BPI=bipolar I disorder, BPII=bipolar II disorder,
NS=not significant.

Table 2
Allele frequencies and genotype of the Asn796Ser polymorphism of the BCR gene and response to lithium treatment

Response to lithium treatment	Allele frequency		χ^2test	Genotype		χ^2test
	Asn	Ser	p value (OR)	Asn/Asn Asn/Ser	Ser/Ser	p value
Responders (n=43)	49 (57.0%)	37 (43.0%)		35 (81.4%)	8 (18.6%)	
Non-responders (n=118)	101 (42.8%)	135 (57.2%)		77 (65.3%)	41 (34.7%)	
			0.024 (1.77)			0.049
Total patients (n=161)	150 (46.6%)	172 (53.4%)		112 (69.6%)	49 (30.4%)	

OR: Odds ratio.

2.2. Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. The genotype of the Asn796Ser SNP (rs140504) of the *BCR* gene was determined by TaqMan 5′-exonuclease allelic discrimination assay, described previously (Hashimoto et al., 2005). Briefly, probes and primers for detection of the polymorphism were: forward primer 5′-AGCTGGACGCTTTGAAGATCA-3′, reverse primer 5′-TGGTGTGCACCTTCTCTCTCT-3′, probe 1 5′-VIC-CCAGATCAAGAATGACAT-MGB-3′, and probe 2 5′-FAM-CCAGATCAAGAGTGACAT-MGB-3′. PCR cycling conditions were: at 95 °C for 10 min, 50 cycles of 92 °C for 15 s and 60 °C for 1 min.

2.3. Statistical analysis

Difference in clinical characteristics between responders and non-responders to lithium treatment was analyzed using the χ^2 tests for categorical variables and t tests for continuous variables. The presence of Hardy–Weinberg equilibrium was examined by using the χ^2 test for goodness of fit. Subsequently, multiple logistic regression analysis was performed to correct background difference between responders and non-responders for lithium treatment. Possible predictors (genotype of the *BCR* gene, subtype of bipolar disorder, age of onset, age at last observation, and gender) were included in the original model. Backward stepwise regression was performed, and p-value greater than 0.10 was used for variable removal. Pearson coefficient of correlation test was used for comparison between recurrence index and clinical variables. The effect of the Asn796Ser SNP on recurrence index was assessed by analysis of variance (ANOVA). All p-values reported are two-tailed. Statistical significance was defined at $p<0.05$.

3. Results

Among 161 patients with BPD, 43 patients were determined as responders and 118 patients as non-responders for the maintenance treatment of lithium. The clinical characteristics sorted by response to lithium treatment and genotype distribution were presented in Table 1. There were significant differences between responders and non-responders in subtype of bipolar disorder (BPI and BPII), age at last observation, and age of onset.

The genotype distributions for the total patients, responders, and non-responders were in Hardy–Weinberg equilibrium (total patients: $\chi^2=0.94$, $df=1$, $p=0.33$; responders: $\chi^2<0.001$, $df=1$, $p=0.98$; non-responders: $\chi^2=0.81$, $df=1$, $p=0.37$). Allele frequencies and genotype distributions of the Asn769Ser polymorphism of the *BCR* gene among responders and non-responders for lithium treatment are presented in Table 2. The Ser796 allele was in excess in the non-responders rather than responders ($\chi^2=5.09$, $df=1$, $p=0.024$; OR 1.77, 95% CI 1.08–2.92). Then, we examined patients homozygous for the Ser796 allele and the Asn796 allele carriers, separately. Patients homozygous for the Ser796 allele were significantly more common in the non-responders than the Asn796 carriers ($\chi^2=3.88$, $df=1$, $p=0.049$; OR 2.33, 95% CI 0.99–5.49). After backward stepwise regression, the final logistic regression model included subtype of bipolar ($p<0.01$), age of onset ($p<0.01$), and genotype which is separated to the Asn796 carrier and homozygous for the Ser796 ($P=0.04$).

We next investigated the association between lithium response using recurrence index and clinical variables in 24 subjects with BPD. The change of recurrence index before to during lithium treatment was not associated with subtype (t=0.79, $df=22$, $p=0.44$), age of onset (correlation coefficient=−0.29, $p=0.17$), duration from onset of illness to lithium treatment (correlation coefficient=0.12, $p=0.57$), duration during treatment (correlation coefficient=0.11, $p=0.60$), or the Asn796Ser SNP ($df=2$, $F=0.03$, $p=0.97$).

We also examined the association between age of onset and recurrence index before lithium treatment, which reflects severity of illness. There was a negative trend between age of onset and recurrence index (correlation coefficient=−0.37, $p=0.074$). Although difference among genotype of Asn796Ser SNP was not statistically significant, the number of Ser796 allele was associated with higher recurrence index before lithium treatment (Asn/Asn=1.63±1.19, Asn/Ser=2.89±0.84, and Ser/Ser=3.23±1.19. $df=2$, $F=0.53$, $p=0.60$). Therefore, the Ser796 allele might also be associated with both early onset and severity of illness, which could result in poorer lithium response.

4. Discussion

We investigated a possible association between the *BCR* gene and the prophylactic effect of lithium treatment in patients with BPD for the first time. As expected, our results suggested that lithium treatment might be less effective in patients homozygous for the Ser796 allele of the *BCR* gene than in patients with the Asn796 allele. In addition, allele frequencies of the Ser796 associated with poorer lithium response were 43.0%

in responders and 57.2% in non-responders. As allele frequency of the Ser796 in healthy subjects in our previous study was 48.1% (Hashimoto et al., 2005), allele frequency of the Ser796 of responders is similar to the general population.

Comparing clinical characteristics of responders and non-responders, there were more BPII patients in non-responder group. Clinical characteristics predicting poorer response to lithium therapy and that of BPII seem to overlap each other, but better lithium response in BPI is not universally accepted. We excluded any Axis I comorbidity in this study. This would leave in more typical bipolar II patients who would be more likely to respond to lithium, however, other clinical factors such as Axis II comorbidity might influence our results. The presence of positive family history of lithium responsive BPD has been reported as indicative of favorable response (Grof et al., 2002). However, it was not assumed that our sample size was enough to investigate this issue because only 8.7% of BPD had positive family history of the same disease in 1st degree relatives (Smoller and Finn, 2003). Therefore, information about family history of lithium response was not collected in this study.

Age at onset was also different between responders and non-responders, and early age of onset was associated with poorer response to lithium treatment in our subjects. This observation is consistent with recent meta-analysis (Kleindienst et al., 2005). As the objective of this study is to examine the association between response to lithium treatment and a SNP in the *BCR* gene, the differences in demographic parameters of responders and non-responders might not be preferable. Therefore, we conducted a multiple logistic regression analysis, and homozygous for the Ser796 allele of the *BCR* gene was still significantly associated with poorer response to lithium treatment.

The evaluation of lithium prophylaxis is considerably difficult because of complex clinical course of BPD, and each researcher has used different methodologies. Although our finding was based on the simple definition, in which lithium responders didn't have any affective recurrences during lithium, one of the limitation of this study is lack of detailed clinical information, e.g. duration from onset of illness to lithium treatment and number of episodes which could be clearly identified before lithium treatment in total subjects. To evaluate lithium efficacy including partial response, calculating recurrence index before and during lithium treatment is used in several researches. This would be a correct measure of lithium prophylaxis, but evaluating mood recurrence accurately before the first contact to mental professionals is difficult. We tried to evaluate lithium response with recurrence index; however, we could examine it in only 24 subjects out of 161 subjects due to the difficulty of collecting this clinical information. We did not found any association between the recurrence index and clinical variables and the SNP in the *BCR* gene, except for the trend between the recurrence index and age of onset. As these results were from subgroup analysis with smaller number, further investigation is needed in a larger sample size.

In this study, the same variant associated with the illness was also associated with poorer outcome. This situation is similar to that of the Val allele of the *BDNF* Val66Met polymorphism (Rybakowski et al., 2005), and it is possible that the *BCR* Ser796 and the *BDNF* Val66 alleles are associated with severer illness presentation. The trend between the recurrence index and age of onset in our subgroup analysis might imply this possibility. In case of the *BDNF* Val66Met SNP, the functional differences arisen from each allele were reported (Eagan et al., 2003). While biological functional of the *BCR* Asn796Ser SNP is still unknown, this SNP may produce functional difference in the brain, like the *BDNF* Val66Met SNP. To speculate this issue, it is noteworthy that this SNP is in the pleckstrin homology (PH) domain of the *BCR*. As PH domain is known for its ability to bind phosphatidylinositol and this binding regulates the activity of PH domain containing protein (Lemmon et al., 2002), signal transduction from inositol cycle to the *BCR* products might be affected by this SNP. As the *BCR* is RhoGAP, this change may influence on the activity of its downstream target, RhoGTPase, which activates many kind of effectors associated with constructing neuronal network, and subsequently influence on neuronal development. Additionally, as inositol cycle is considered as one of therapeutic targets of lithium (Harwood, 2005), this SNP could alter the clinical efficacy of lithium. To understand the mechanism of our findings, it is worth investigating whether the Asn796Ser SNP alters the binding ability of PH domain to inositol.

5. Conclusion

This is the first report demonstrating that long-term lithium treatment may be less effective in BPD patients homozygous for Ser796 allele of the *BCR* gene than in patients with the Asn796 allele. The limitations of this study are retrospective design without placebo control group, small sample size, and lack of clinical information such as presence of rapid cycling and/or psychotic symptoms, and detailed lithium levels. Further investigations are needed to confirm our findings.

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Regulation of Notch Signaling by Dynamic Changes in the Precision of S3 Cleavage of Notch-1▽†

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Intramembrane proteolysis by presenilin-dependent γ-secretase produces the Notch intracellular cytoplasmic domain (NCID) and Alzheimer disease-associated amyloid-β. Here, we show that upon Notch signaling the intracellular domain of Notch-1 is cleaved into two distinct types of NICD species due to diversity in the site of S3 cleavage. Consistent with the N-end rule, the S3-V cleavage produces stable NICD with Val at the N terminus, whereas the S3-S/S3-L cleavage generates unstable NICD with Ser/Leu at the N terminus. Moreover, intracellular Notch signal transmission with unstable NICDs is much weaker than that with stable NICD. Importantly, the extent of endocytosis in target cells affects the relative production ratio of the two types of NICD, which changes in parallel with Notch signaling. Surprisingly, substantial amounts of unstable NICD species are generated from the Val→Gly and the Lys→Arg mutants, which have been reported to decrease S3 cleavage efficiency in cultured cells. Thus, we suggest that the existence of two distinct types of NICD points to a novel aspect of the intracellular signaling and that changes in the precision of S3 cleavage play an important role in the process of conversion from extracellular to intracellular Notch signaling.

Presenilin (PS)-dependent γ-secretase (PS/γ-secretase) mediates the degradation of transmembrane domains (TMs) in many type 1 receptors, including Notch and β-amyloid protein precursor (βAPP) (18, 52). Degradation of these receptors is characterized by sequential endoproteolysis: following shedding by cleavage in the extracellular juxtamembrane region, the receptors undergo PS-dependent intramembrane proteolysis, releasing amyloid-β (Aβ)-like peptides and intracellular cytoplasmic domains (ICDs) (5, 14). At least in the cases of βAPP, Notch, and CD44, cleavages of the C termini of Aβ-like peptides and of the N termini of ICDs in the TM are distinct. This process of cleavage at two sites is known as "dual cleavage" (33). The process of Aβ generation has been intensively studied, and it is suggested that Aβ is released by a series of sequential cleavages followed by the ICD generation (25, 36, 53). An unusual characteristic of this intramembrane proteolysis is that some of the cleavage sites can vary (42). The precision of cleavage can therefore be defined as the ratio of the cleavage at each site. For example, PS-dependent cleavage of βAPP at the γ site, which is associated with Alzheimer disease (AD), occurs mainly at residue 40 (γ40), producing Aβ40, and at residue 42 (γ42), producing Aβ42. A small increase in the proportion of γ42 to γ40 cleavage is consistently observed in many familial AD (FAD)-associated PS or βAPP mutants (42), but it is unclear whether such changes in the precision of PS-dependent intramembrane proteolysis have any biological effects.

Notch signaling, which is essential for development, is a type of local-cell signaling that participates in neurodegeneration and tumorigenesis (1). The canonical Notch pathway is mediated by the regulated intramembrane proteolysis pathway, in which Notch receptors undergo ligand-dependent sequential endoproteolysis via a series of enzymes, including PS/γ-secretase (8). The Notch-1 ICD (NICD), which is produced by PS/γ-secretase-mediated cleavage at site 3 (S3), translocates to the nucleus and participates in transactivation of target genes (40). Elimination of PS function results in the Notch phenotype, which includes disruption of segmentation during the development of many kinds of animals, demonstrating the importance of NICD generation (41).

The intensity of Notch signaling is crucial for cell fate decisions. For example, Notch haplo-insufficiency causes the "notched-wing" phenotype in *Drosophila* (9). Reduced Notch activity favors the γδ T-cell fate over the αβ T-cell fate, whereas a constitutively activated form of Notch produces a reciprocal phenotype (48). The endocytosis of Notch and its ligands plays a key role in the regulation of the signaling intensity (40), but the biochemical aspects regulating this process have not been well studied. N-terminal amino acid se-

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quencing revealed that S3 in mouse Notch-1 lies between Gly1743 and Val1744 (murine Notch-1 numbering) (39). Whether the site of S3 cleavage can vary has not been examined previously.

In this study, we found that there is diversity in the site of S3 cleavage, resulting in the production of two types of NICD with apparently distinct stability and ability to transmit Notch signaling in cultured cells. Our results suggest that the precision of PS/γ-secretase-mediated cleavage is important for determining the intensity of Notch signaling.

MATERIALS AND METHODS

Antibodies. To generate affinity-purified polyclonal N-terminal capping antibodies to NICD-S (anti-NT-S), rabbits were immunized with a synthetic peptide (SRKRR) corresponding to the N terminus of NICD-S(+3). We also prepared two kinds of affinity columns in which the N-terminal peptide of NICD-V (VLLSRKRR) or NICD-S (SRKRR) was conjugated to Sepharose 4B (Amersham). We isolated the fraction of the anti-NT-S antiserum that bound to the NICD-S(+3) column but not the NICD-V column (32). Anti-NT-L antiserum was raised against a synthetic peptide (LLSRKRR) corresponding to the N terminus of NICD-L(+1) and then purified by affinity chromatography on Sepharose 4B conjugated to the N-terminal peptide of NICD-L (LLSRKRR), followed by a second step of affinity chromatography on Sepharose 4B conjugated to the NICD-S peptide (SRKRR). Other antibodies were purchased from commercial sources as follows: anti-NT-V (V1744 antibody) from Cell Signaling; antinicastrin and antibody mN1A against Notch-1 from Sigma-Aldrich; antibody H114 against Jagged-1 and antitubulin from Santa Cruz Biotechnology; anti-early endosome antigen 1 and anti-GM130 from BD Transduction Laboratories; antibody 12CA5 against the HA epitope from Roche Diagnostics, Inc.; antibody 9E10 against the myc epitope from Zymed; and anti-Na-K ATPase from Upstate Biotechnology.

cDNA constructs. The cDNA encoding the mouse Notch-1 variant NEXT was previously described (33). NEXTΔC was generated by PCR-based mutagenesis using the QuikChange-II kit (Stratagene) with NEXT cDNA as a template. The mutant versions of NEXT and NEXTΔC were generated using the same kit. To generate expression constructs for polypeptides NICD-V, NICD-L (+1), and NICD-S (+3), cDNAs encoding Val, Leu(+1), and Ser(+3) as the N termini were subcloned into the pASK-IBA6 vector (IBA). *HES-1*-luc (a kind gift from Alain Israel) (16) and pGa981-6 (a kind gift from Georg W. Bornkamm) (21) were used as described. For more sensitive detection of *HES-1* promoter transactivation, we newly generated a *hairy and enhancer of split-Y* (*HES-Y*) construct containing four sequential RBP-Jκ binding sites in the *HES-1* promoter region.

Cell culture. We generated HEK293 cells expressing PS1 R278I (a kind gift from M. Nishimura) (27) or PS1 G384A (a kind gift from H. Steiner) (44). HEK293 cells expressing either wild-type (wt) or mutants of PS1 were previously described (31). These cells were transfected with wt, mutant NEXT, or mutant NEXTΔC. HeLa cells expressing Dyn-1 K44A (a kind gift from S. Schmid) were used as described and stably transfected with NEXT or NEXTΔC. CHO(r) cells (a gift from S. Shirahata) (29) were stably transfected with mouse Notch-1 or Jagged-1.

Cell-free Notch-1 cleavage assay. To obtain crude membrane fractions (CMFs), cells were homogenized in buffer (0.25 M sucrose and 10 mM HEPES, pH 7.4) containing a protease inhibitor cocktail (Roche), followed by centrifugation at 1,000 × *g* for 5 min. The postnuclear supernatant was further centrifuged at 100,000 × *g* for 30 min, and the resulting pellet was collected. This CMF was resuspended in 150 mM sodium citrate buffer (pH 6.4) containing a 4× concentration of protease inhibitor cocktail (Sigma-Aldrich) and 5 mM 1,10-phenanthroline (Sigma-Aldrich), incubated for 20 min at 37°C, and then centrifuged at 100,000 × *g* for 30 min (11).

Pulse-chase experiments. Pulse-chase experiments were performed as described previously (11, 30, 31, 33, 34).

Immunoprecipitation/MALDI-TOF MS. Immunoprecipitation and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) analysis was carried out as described previously (11, 31, 33). The heights of the MS peaks and molecular weights were calibrated using angiotensin and bovine insulin β-chain as standards (Sigma-Aldrich).

Immunoprecipitation-immunoblotting and immunoprecipitation-autoradiography. Metabolically labeled or unlabeled lysates were lysed in radioimmunoprecipitation assay buffer (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) containing a protease inhibitor mix (Sigma-Aldrich). The cell lysates were centrifuged at 10,000 × *g* for 15 min, and the supernatant fractions were immunoprecipitated as indicated. Following 8% Tris-glycine (Tefco) or 10 to 20% Tris-Tricine (Invitrogen) SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the gels were either transferred to a polyvinylidene difluoride membrane and probed with the indicated antibodies or dried and analyzed by autoradiography. To quantitatively measure the levels of NICD species in cultured cells, several doses of each NICD polypeptide were separated together with samples by SDS-PAGE and analyzed by immunoblotting with the corresponding N-terminal capping antibody. The chemiluminescence intensities were measured using an LAS3000 scanner, followed by analysis with Multi Gauge Ver3.0 software (Fuji Film). Biotinylated transferrin was semiquantitatively measured by chemiluminescence using the scanner, followed by analysis with the software.

Cell-cell association assay. For the detection of de novo NICD species, CHO(r) cells stably expressing Notch-1 were grown to confluence in 150-mm dishes (2×10^7 cells per dish) in triplicate. Next, 3×10^7 CHO(r) cells stably expressing Jagged-1 were spread over the Notch-1-expressing cells. After 8 h of coculture, the cells were collected. For the reporter assay, the procedure was the same, although it was carried out in a 12-well plate and the number of cells was reduced accordingly.

cDNA transfection and reporter assay. To examine the intensity of Notch signaling, we used a dual luciferase reporter assay system (Promega) as described by the manufacturer (31). Briefly, cells expressing Notch-1 or its derivatives in a 12-well plate were transiently transfected with 125 ng of *HES-Y* or pGa981-6 and 1.25 ng of the control *Renilla* luciferase reporter plasmid pRL-TK. The reporter assay was performed on the next day. Dyn-1 K44A expression was induced with various concentrations of tetracycline 24 h prior to transfection with *HES-Y*. Cell-cell association was performed 24 h after transfection with *HES-Y*.

Preparation of nuclear extract from mouse tissues. A nuclear complex co-IP kit (Active Motif) was used to obtain nuclear extracts from C57BL/6 (Japan SLC) mouse tissues. Homogenized adult mouse brain or fetal mouse tissues without internal organs (embryonic day 12) were treated according to the manufacturer's instructions. Subsequently, the nuclear extracts were diluted using the immunoprecipitation buffer included in the kit, precleared three times with protein G- or protein A-Sepharose, and immunoprecipitated according to the manufacturer's instructions.

Purification of polypeptides. NICD-V, NICD-L(+1), and NICD-S(+3) polypeptides fused with *strept*-tag-II followed by an N-terminal factor Xa cleavage site were obtained by transforming *Escherichia coli* (BL21) with pASK-IBA6 (IBA) encoding each polypeptide. Briefly, after the expression was induced, the cells were collected by centrifugation at 4,500 × *g* for 12 min, resuspended in ice-cold TSE buffer (10 mM Tris-HCl [pH 7.4], 20% sucrose, and 2.5 mM Na-EDTA), and then incubated on ice for 10 min. The cells were again collected by centrifugation, resuspended in ice-cold water, incubated for 10 min, briefly sonicated, and sedimented by centrifugation at 14,000 × *g* for 15 min (osmotic shock fractionation). The supernatant was passed through a Strep-Tactin Sepharose column (IBA). Bound polypeptides were eluted with phosphate-buffered saline containing 2.5 mM desthiobiotin. Eluted polypeptides were treated with factor Xa (Sigma-Aldrich), and the solution was passed through the column again to remove uncleaved polypeptide (34). The purity of the polypeptides was confirmed by 6% Tris-glycine SDS-PAGE, followed by staining with Coomassie brilliant blue.

Loading of polypeptides. The polypeptides obtained as described above were loaded into cells using Chariot protein transfection reagent (Active Motif) according to the manufacturer's instructions. Briefly, cells were grown and transfected with reporter genes in a 12-well plate. The cells were then loaded with 5 μg of each polypeptide or bovine serum albumin (BSA) along with 0.5 μg of β-galactosidase, using 15 μl of Chariot reagent per well. Finally, the cells were stained for β-galactosidase or used for the reporter assay.

In vitro degradation assay. NICD polypeptides (0.2 μg) were mixed with 60 μl of fresh rabbit reticulocyte lysate (Promega) and incubated at 37°C. Clasto-lactacystin (10 μM), MG262 (100 nM), and 4-hydroxy-5-iodo-3-nitrophenyl-acetyl-Leu-Leu-leucinal-vinyl sulfone (NLVS) (10 μM) were added to inhibit the action of the proteasome.

Transferrin uptake assay. To estimate the rate of endocytosis, the levels of internalized and surface-bound biotinylated-transferrin were measured as described previously (11).

Subcellular fractionation. Linear gradients of 2.5% to 25% iodixanol (Optiprep; Axis-Shield) were prepared, and fractionation was performed as previously described (11).

Statistical analysis. Experiments were performed at least three times unless otherwise indicated. Representative results are shown for cell-free immunoprecipitation/MALDI-TOF MS, immunoblotting, immunoprecipitation-autoradiog-

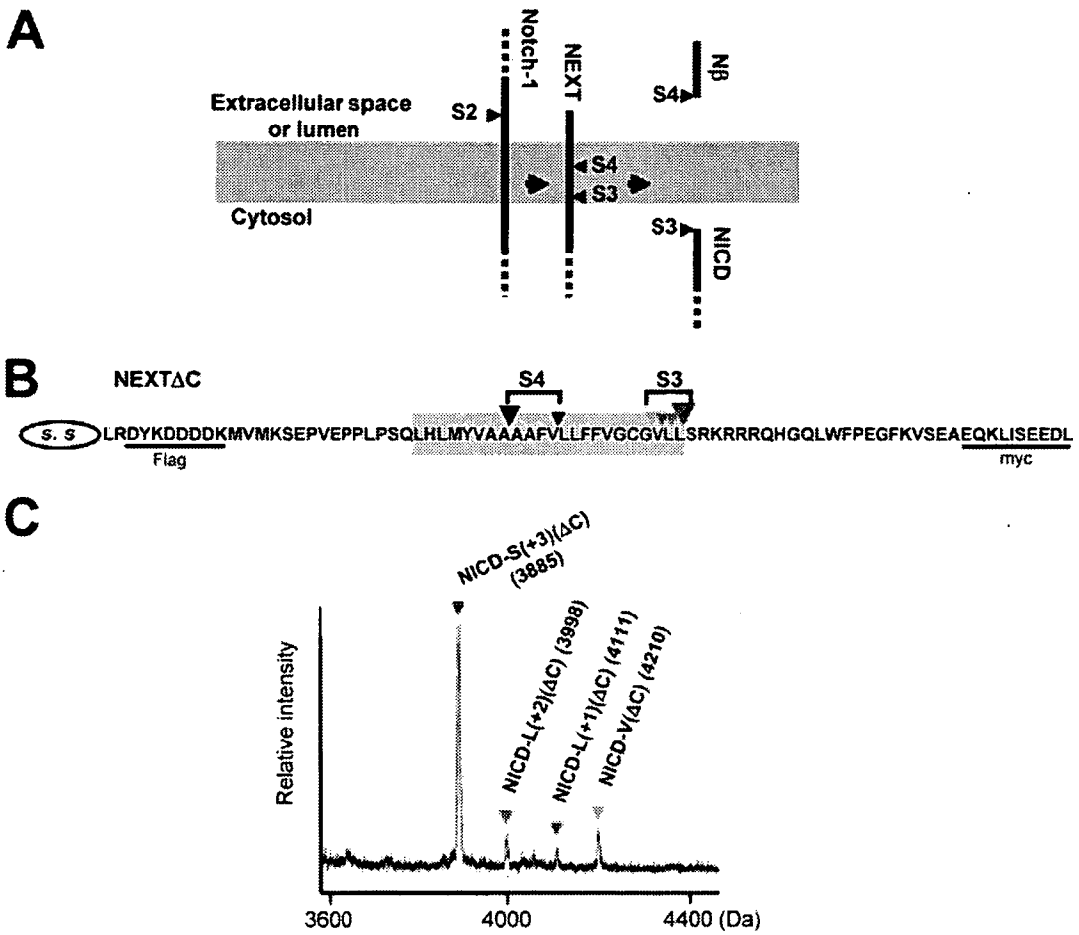

FIG. 1. MALDI-TOF MS analysis of NICD(ΔC) produced by the cell-free Notch-1 cleavage assay. (A) Schematic representation of sequential endoproteolysis of Notch-1. S2 to S4 and the gray area represent the proteolytic sites and the putative TM, respectively. (B) Schematic representation of the NEXTΔC construct used in the cell-free Notch-1 cleavage assay. Colored inverted triangles show the S3 and S4 proteolytic sites. SS, signal sequence. (C) MS spectrum of de novo NICD(ΔC) generated in the cell-free assay. CMF was derived from K293 cells stably expressing NEXTΔC. The molecular mass of each species is indicated. To inhibit degradation by proteases other than aspartyl proteases, BSA and a mixture of metallo-, serine, and cysteine protease inhibitors were added to the cell-free assay buffer. Colored inverted triangles indicate the NICD(ΔC) species produced by cleavage at the sites shown in panel B.

raphy, and immunocytochemistry. The statistical significance of differences was determined by Student's *t* test.

RESULTS

A cell-free Notch-1 cleavage assay indicates diversity in the site of S3 cleavage. The cleavage of the Notch-1 TM occurs at least at two sites, one at S3, which determines the N terminus of intracellularly liberated NICD (39), and the other at S4, which determines the C terminus of extracellularly secreted Nβ (Fig. 1A) (31, 33). We first examined the diversity of the S3 cleavage site. We constructed NEXTΔC, a mouse Notch-1 derivative that lacks the majority of its extracellular and intracellular domains (Fig. 1B), and we established a cell-free Notch-1 cleavage assay using the CMF from cells stably expressing this construct (11). The de novo-generated NICD(ΔC) was immunoprecipitated with anti-myc antibodies and then analyzed by MALDI-TOF MS (Fig. 1C). Strikingly, proteolysis at S3 did not occur at a unique site but rather occurred at multiple sites, as indicated by the presence of multiple sizes of NICD(ΔC) (see Table S1 in the supplemental material). Specifically, proteolysis at S3 occurred at the following sites: S3-L(+1), between Val1744 and Leu1745; S3-L(+2), between Leu1745 and Leu1746; S3-S(+3), between Leu1746 and Ser1747; and the previously reported S3-V, between Gly1743 and Val1744 (39) (Fig. 1B; see Fig. S1A in the supplemental material). Unexpectedly, the highest peak was for NICD-S(+3)(ΔC) rather than NICD-V(ΔC) (Fig. 1C), suggesting that S3-S(+3) is the major site of S3 cleavage under these assay conditions. Addition of the PS/γ-secretase inhibitors eliminated the cleavage at both S3-V and S3-S(+3) (see Fig. S1B in the supplemental material). Moreover, we did not observe generation of these shorter NICD(ΔC) species from the longer NICD(ΔC) (see Fig. S1C in the supplemental material). Therefore, the results are consistent with the possibility that all the fragments are produced by PS-dependent S3 cleavage in the Notch-1 TM.

Diversity in the site of S3 cleavage in living cells. To identify the N terminus of NICD molecules in vivo, we prepared two N-terminal capping antibodies, anti-NT-V (anti-V1744) and anti-NT-S (32), and corresponding recombinant NICD species