Leung and Chue, 2000). Generally, male patients with schizophrenia tend to be more severely ill than their female counterparts as shown by earlier age of onset, poorer premorbid functioning, severer cognitive deficits, and higher risk of having a deficit state (Castle et al., 1993; Leung and Chue, 2000). Some of these gender differences might reflect the gender difference in personality in the present study, in which male patients apparently showed more unique personality profiles than female patients.

4.3. Relationships between personality and symptom dimensions

In the present study, since TCI dimensions were somewhat correlated with schizophrenic symptoms and dosage of antipsychotics, personality might be affected by illness severity. For instance, ST was positively correlated with positive symptoms and dosage of antipsychotics, suggesting that this personality dimension could be substantially related to the severity of positive symptoms. This positive correlation between ST and psychotic symptoms has also been found in the precedent study (Guillem et al., 2002) which used the three-dimensional model of Andreasen et al. (1995) and the five-dimension model of Toomey et al. (1997) for assessment of symptoms. In addition, Guillem et al. (2002) have reported that psychotic symptoms are positively correlated with NS and negatively with SD, both of which were not replicated in the present study. Instead, in the present study negative symptoms were negatively correlated with RD, PS and CO. These correlations in the present study appear to be plausible, given that the negative subscale of PANSS is composed of items which assess, for example, "blunted affect", "passive social withdrawal", and "poor rapport". These relationships observed between personality and symptom dimensions were in support of our hypothesis (iii). The inconsistencies between studies might be due in part to the differential instruments of symptom assessment used, and require further investigations.

4.4. Strengths and limitations of the study

A major advantage in this study was that we examined personality by using the TCI, which has a number of merits in personality research that have already been mentioned in the present report. Sample size of the present study was the largest of the four personality studies of schizophrenia where the TCI was used (Guillem et al., 2002; Boeker et al., 2006; Calvo de Padilla et al., 2006). Moreover, our patients and controls were matched for age and gender, and male and female patients were similar to each other regarding all clinical variables examined, both of which made further comparisons by gender relatively free from confounders. On the other hand, education was significantly different between patients and controls or control males and females, but the lowered education in schizophrenia could be closely related to the illness itself. It should also be noted that in Japan it is common for men to achieve higher educational status than women. Thus our subjects, both patients and controls, are likely to be representative of the general Japanese population in terms of education.

There are several limitations to this study. First, its cross-sectional nature does not permit any definite conclusions as to whether personality traits we found to be altered in schizophrenia are premorbid ones or the results of changes after illness onset. Second, personality assessment was based on self-report, thus not objective. However, our results that showed moderate correlations between TCI and PANSS scores might suggest that the results of subjective personality assessment with the questionnaire were corroborated by those of objective symptom assessment. Third, since our sampling was not community-based random sampling, it is possible that certain sampling biases existed; people who had high "novelty seeking", for example, were likely to become interested in this study. Finally, our patients with schizophrenia were chronic, which precluded extrapolation of the results to recent-onset patients.

4.5. Conclusions

The present findings indicate that patients with chronic schizophrenia have pervasively altered personality profile as measured by TCI which is in line with previous studies, and male patients may undergo even more pronounced personality alteration than female patients when both of them are compared to healthy people.

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Genetic Variations of Human Neuropsin Gene and Psychiatric Disorders: Polymorphism Screening and Possible Association with Bipolar Disorder and Cognitive Functions

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Human neuropsin (NP) (hNP) has been implicated in the progressive change of cognitive abilities during primate evolution. The hNP gene maps to chromosome 19q13, a region reportedly linked to schizophrenia and bipolar disorder. Therefore, hNP is a functional and positional candidate gene for association with schizophrenia, mood disorders, and cognitive ability. Polymorphism screening was performed for the entire hNP gene. The core promoter region was determined and whether or not transcriptional activity alters in an allele-dependent manner was examined by using the dual-luciferase system. Allelic and genotypic distributions of five single-nucleotide polymorphisms (SNPs) were compared between patients with schizophrenia ($n=439$), major depression ($n=409$), bipolar disorder ($n=207$), and controls ($n=727$). A possible association of the hNP genotype with memory index (assessed with Wechsler Memory Scale, revised, WMS-R) and intelligence quotient (IQ assessed with Wechsler Adult Intelligence Scale, revised; WAIS-R) was examined in healthy controls ($n=166$). A total of 28 SNPs, including nine novel SNPs, were identified. No significant effects on transcriptional activity were observed for SNPs in the promoter region. A significant allelic association was found between several SNPs and bipolar disorder (for SNP23 at the 3′ regulatory region; odds ratio 1.48, 95% confidential interval 1.16–1.88, $P=0.0015$). However, such an association was not detected for schizophrenia or depression. Significant differences were observed between SNP23 and attention/concentration sub-scale score of WMS-R ($P=0.016$) and verbal IQ ($P<0.001$). Genetic variation of the hNP gene may contribute to molecular mechanisms of bipolar disorder and some aspects of memory and intelligence.
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Keywords: neuropsin; polymorphism screening; association study; bipolar disorder; memory; intelligence

INTRODUCTION

Neuropsin (NP, MIM: 605644), also called as kallikrein 8 (KLK8), is one of the secreted-type serine proteases, which was first cloned by our group in mice (Chen *et al*, 1995). NP mRNA is expressed specifically in the limbic system of mouse brain and is localized at the highest concentration in pyramidal neurons of the hippocampal CA1-3 sub-fields. Direct hippocampal stimulation and kindling induced by amygdaloid stimulation caused a significant bilateral change in NP mRNA level in the hippocampal pyramidal neurons. The activity-dependent changes and the specific localization indicate that NP is involved in hippocampal plasticity (Chen *et al*, 1995). Indeed, NP has a regulatory effect on Schaffer-collateral at the early phase of long-term potentiation (LTP) (Komai *et al*, 2000). Mice lacking NP were significantly impaired in the Morris water maze and Y maze, suggesting that NP has an important role in learning and memory (Tamura *et al*, 2006). The human NP (hNP) gene was cloned by Yoshida *et al* (1998), and then localized to chromosome 19q13.3–q13.4 (Gan *et al*, 2000; Harvey *et al*, 2000). It consists of six exons and the first exon is non-translational. Four alternative splicing variants have been identified (Mitsui *et al*, 1999; Magklara *et al*, 2001). The regular form is called type1, and type 2 contains a 135-bp

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insertion of 5′ upstream region of exon 3 (Mitsui *et al*, 1999). Interestingly, type 2 is a hominoid-specific splicing form (Li *et al*, 2004) and is expressed as abundantly as the type 1 in human brain (Mitsui *et al*, 1999). These findings points to the possibility that type 2 hNP may contribute to progressive change of cognitive abilities during primate evolution. Moreover, dysfunctions in hNP may be involved in psychiatric diseases of cognitive abilities, including schizophrenia and mood disorders.

Family, twin, and adoption studies clearly suggest that genetic components play an important role in the pathogenesis of schizophrenia and mood disorders (reviewed by Shih *et al*, 2004). These psychiatric diseases demonstrate substantial cognitive deficits such as learning and memory (reviewed by Sharma and Antonova, 2003; Robinson *et al*, 2006; Green, 2006). A genome screen of linkage with bipolar disorder pedigrees provided evidence for susceptibility locus on chromosome 19q13 (Badenhop *et al*, 2002). Another genome scan in schizophrenia and bipolar pedigrees obtained an LOD ratio score of 1.5 at 19q13 in schizophrenic families (Macgregor *et al*, 2004). Therefore, the hNP gene is a good candidate gene for association with schizophrenia and mood disorders. Here we performed, for the first time, a polymorphism screening and association analysis of the hNP gene with schizophrenia, major depression, and bipolar disorder in a Japanese sample. A possible association of hNP with memory and intelligence in healthy subjects was also examined. In addition, we determined a core promoter region of the hNP gene and examined whether transcriptional activity varies in an allele-dependent manner.

MATERIALS AND METHODS

Subjects

Subjects for the association study were 439 patients with schizophrenia (240 males, mean age of 44.6 years (SD 14.0)), 409 patients with major depression (136 males, 53.3 years (15.9)), 207 patients with bipolar disorder (80 males, 50.2 years (14.7)), and 727 healthy controls (324 males, 43.5 years (16.4)). Among these, 104 patients with bipolar disorder and 108 controls were recruited around Shiga prefecture, approximately 350 km to the west of Tokyo, while the remaining 1570 subjects were recruited around Tokyo. Consensus diagnosis by at least two psychiatrists, one of whom was in charge of the patients, was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association, 1994), on the basis of unstructured interviews and information from medical records. Control subjects were healthy volunteers who had no current or past contact to psychiatric services. Among them 213 controls were screened by the Japanese version of the Mini-International Neuropsychiatric Interview (Sheehan *et al*, 1998; Otsubo *et al*, 2005) by a research psychiatrist, whereas the remaining controls were not screened by such a structured interview. Participants were excluded if they had prior medical histories of central nervous system disease or severe head injury, or if they met the criteria for substance abuse or dependence, or mental retardation. All subjects were biologically unrelated Japanese. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethics committees.

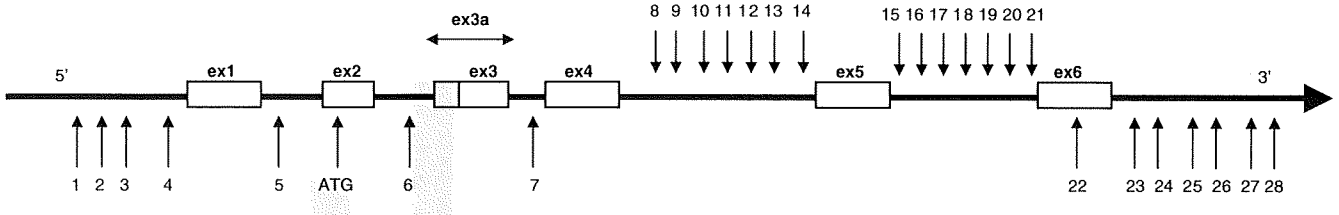
Neuropsychological Test Measures

Among controls, 166 (53 males, 37.6 years (12.4)) were subject to memory and intelligence tests to detect possible association with the hNP genotype. These individuals were all screened by the Mini-International Neuropsychiatric Interview with respect to their psychiatric history and confirmed that they had no current or past history of psychiatric illness. To assess memory and intelligence, Japanese full versions of the Wechsler Memory Scale-Revised (WMS-R) (Sugishita, 2001; Wechsler, 1987) and the Wechsler Adult Intelligence Scale-Revised (WAIS-R) (WAIS-R, Shinagawa *et al*, 1990; Wechsler, 1981), respectively, were administered. Testing and scoring were performed by psychologists who were blind to genotypic data.

Polymorphism Screening and Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The genomic structure of hNP was determined from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and the University of California at Santa Cruz (UCSC) database (http://genome.ucsc.edu/cgi-bin/hgBlat). To screen for polymorphisms, we used direct sequencing with the Genome Lab-DTCS (Dye Terminator Cycle Sequencing) kit and CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). The entire 7428-bp genomic region containing all the exons, introns, the 1078-bp 5′ flanking region upstream to exon 1, and the 655-bp 3′ flanking region downstream to exon 6 were amplified from the genomic DNA of 24 randomly selected schizophrenic subjects. Sequences of 24 sets of primers for the polymorphism screening are listed in Supplementary Table S1.

The examined 7428-bp region seemed to constitute of single haplotype blocks (Supplementary Figure S1). We genotyped five single-nucleotide polymorphisms (SNPs) using TaqMan 5′-exonuclease allelic discrimination assay. They were A-807>G (ss73688625, SNP3), G-658>C (rs1722550, SNP4), IVS2-101T>A (rs1701947, SNP6), IVS3-10A>G (rs1701946, SNP7), and A5229>G (rs1612902, SNP23) (Figure 1). SNPs 3 and 4 were chosen from the 5′ regulatory region since they may have some effects on transcriptional activity, and SNPs 1, 2, 4, and 5 were in absolute linkage disequilibrium (LD) (ie, genotypes were completely the same) with each other. SNPs 6 and 7 were chosen because they were SNPs located close to the splicing sites of exon 3a (ie, an exon specific to type 2 hNP) and exon 4, respectively, and may have some effects on splicing. SNP23 was chosen from the 3′ region, since SNPs15, 16, 17, 19, 21, 23, 24, 25, 27, and 28 were in absolute LD with each other. TaqMan probes and Universal PCR master mix were obtained from Applied Biosystems (Foster City, CA). Thermal cycling conditions for polymerase chain reaction (PCR) were 1 cycle at 95°C for 10 min followed by 50 cycles of 92°C for 15 s and 60°C for 1 min. After



1. rs2569456	g.G-1302>T	15. rs1654539	g. IVS5+106G>A
2. rs6509509	g.C-948>T	16. rs1722559	g. IVS5+184C>T
3. ss73688625	g.A-807>G	17. rs1701914	g. IVS5+187T>C
4. rs1722550	g.G-658>C	18. rs1722560	g. IVS5+673T>G
5. ss73688633	g.IVS1+25,26 ins/del CA	19. rs1722561	g. IVS5+764T>C
6. rs1701947	g.IVS2-236T>A (exon3)	20. rs1701915	g. IVS5-442A>G
7. rs1701946	g.IVS3-10A>G	21. rs1627239	g. IVS5-355G>T
8. ss73688628	g.IVS4+596G>A	22. ss73688626	g. G5047>A (Val 286 Ile)
9. rs7245714	g.IVS4+866C>T	23. rs1612902	g. A5229>G
10. ss73688629	g.IVS4+1034C>T	24. rs1654542	g. T5308>C
11. ss73688630	g.IVS4-951A>G	25. rs1654543	g. A5435>G
12. rs12610313	g.IVS4-624C>A	26. ss73688627	g.A5579>G
13. ss73688631	g.IVS4-585C>T	27. rs1897605	g. T5646>C
14. ss73688632	g.IVS4-306T>C	28. rs1897606	g. C5777>T

Figure 1 Genomic structure and identified polymorphisms in the human NP gene. A total of 28 SNPs, including one insertion/deletion (ins/del) polymorphism, were identified. The A of the translational start ATG is designated + 1. Nine SNPs were novel and have been registered in the dbSNP (ss-tagged numbers). ex, exon; ex3a, exon3 in hNP type2.

amplification, the allele-specific fluorescence was measured with ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems). Genotype data were read blind to the case-control status. Ambiguous genotype data were not included in the analysis.

Promoter Assay in Primary Cultured Neurons

Primary dissociated cultures were prepared from the brain cortex of postnatal 2-day-old rats (SLC, Shizuoka, Japan) as described previously (Numakawa *et al*, 2002). To generate plasmids for luciferase gene reporter assay, two differentially sized (964 and 128 bp) fragments of the 5′ flanking region of hNP were amplified by PCR with primers 5′-CGA CGCGTGCGTGTGCTGGGTTTGAA-3′ (forward) and 5′-GA AGATCTCTAGAGCCTGGGGAGCTTCT-3′ (reverse) for the 964-bp fragment, and 5′-CGACGCGTCCTCCTCTCCCTAGC CTCAG-3′ (forward) and 5′-GAAGATCTCTAGAGCCTGGG GAGCTTCT 3′ (reverse) for the 128-bp fragment. These primers were designed to incorporate *Mlu*I (forward) and *Bgl*II (reverse) restriction sites, and the PCR product was inserted into the multiple cloning site upstream of the luciferase coding region in the pGL3-Basic vector (Promega, Madison, WI). The inserted sequence was confirmed with the auto sequencer CEQ8000 in both directions using primers 5′-TCTCCATCAAAACAAAACGAA-3′and 5′-TTCC ATCTTCCAGCGGATA-3′.

Among the four SNPs (SNPs 1–4; see Figure 1) in the 5′ upstream region (ie, putative promoter region) of the hNP gene, the genotypes of SNPs 1, 2, and 4 were completely the same for all the 24 schizophrenic subjects, and we found a significant association of bipolar disorder with SNP4 but not SNP3 (see results). In addition, haplotypes containing the A allele (the major allele), but not the G-allele, of SNP3 showed some evidence for association with bipolar disorder in haplotype analysis (see Table 2). We therefore made two allele-specific promoter fragments (haplotypes consisting of SNPs 1-2-3-4 were G-C-A-G and T-T-A-C) of 964- and 128 bp upstream from the transcription initiation site, which were subject to the luciferase reporter gene assay. The plasmid constructs were transfected into cultured neurons at 5 days *in vitro*. Cells on 24-well plates were co-transfected with 3200 ng of pGL3-Basic firefly luciferase reporter vectors, which included allele-specific promoter fragments of 964 and 128 bp, and 100 ng of phRL-TK *Renilla* luciferase vector (Promega, Tokyo, Japan) as an internal control using Lipofectamine 2000 regent (Invitrogen, Tokyo, Japan). As negative control, an empty pGL3-Basic vector was simultaneously transfected in all the experiments. At 24 h after transfection, luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany), as described previously (Tadokoro *et al*, 2004; Okada *et al*, 2006). Firefly and *Renilla* luciferase activities were quantified sequentially as relative light unit (RLU) by addition of their respective substrates according to the protocol of the supplier. The ratio of firefly RLU to *Renilla* RLU of each sample was automatically computed. The activity of each construct was expressed at the relative value compared with that of pGL3-Promoter (as a positive control), and these relative values were computed by *t*-test. Primary cultured cells were prepared six times and transfection was performed quadruplicate for each cell culture.

Statistical Analysis

Deviations of genotype distributions from Hardy–Weinberg equilibrium were assessed with χ^2-test for goodness of fit. Genotype and allele distributions of each SNP were compared between patients and controls using χ^2-test for independence. The association of the hNP genotype with

memory and intelligence was examined by multiple analysis of variance (MANOVA) controlling for possible confounders (age, sex, and education years). These tests were performed with the SPSS software version 11 (SPSS Japan, Tokyo, Japan). The LD (D') between polymorphisms was examined using the Haploview program (http://www.broad.mit.edu/mpg/haploview/) (Barrett *et al*, 2005) and haplotype-based association analyses were performed with COCAPHASE software version 2.4 (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/; Dudbridge *et al*, 2000). The expectation-maximization (EM) and 'droprare' options were used. Haplotypes with frequencies less than 3% were considered to be rare. We examined associations by permutation procedure (10 000 replications) to determine the empirical significance. All P-values reported are two-tailed. Statistical significance was considered when $P<0.05$.

RESULTS

Polymorphism Identification and Genotyping

The 7428-bp genomic region containing all the exons, introns, 5′ flanking, and 3′ flanking regions of hNP were screened for polymorphisms in 24 schizophrenic patients. A total of 28 SNPs, including one insertion/deletion (ins/del) polymorphism, were identified (Figure 1). Among them 19 SNPs had already been listed in the NCBI dbSNP database, whereas nine SNPs were novel. Four SNPs were located in 5′ upstream, one SNP in exon 6, six SNPs in 3′ downstream, and the remaining 17 polymorphisms in introns. There was only one SNP that resulted in an amino-acid change, SNP22 (G5047>A; Val286Ile: the number of the amino acid is according to NP_653088), which gave rise to a restriction site for *Acy*I and was located at an evolutionarily conserved (rodents through humans) residue. This non-synonymous polymorphism was found in only one schizophrenic patient. Additional genotyping was performed for 178 individuals with schizophrenia; however, there was no individual carrying the 286Ile allele, indicating that this amino-acid change is a rare mutation. The LD between SNPs is shown in Supplementary Figure S1, indicating that the entire genomic region consists of single haplotype block. Genotypes for SNPs 1, 2, 4, and 5, those for SNPs 8 and 9, those for SNPs 10 and 13, and those for SNPs15, 16, 17, 19, 21, 23, 24, 25, 27, and 28, respectively, were completely the same as each other for the 24 individuals.

Promoter Assay

We identified four SNPs in the 1078-bp 5′ upstream region of the hNP gene, and SNPs 1, 2, and 4 were found to be associated with bipolar disorder, memory, and intelligence quotient (IQ) (see below). Furthermore, to our knowledge, there is no information in the literature on the location of core promoter of the hNP gene. We therefore performed a promoter assay using the dual-luciferase system (Promega) in rat cultured cortical neurons and examined whether transcriptional activity alters in an allele-dependent manner. As shown in Figure 2a, pGL3-Basic vectors containing 128- and 964-bp fragments, which consisted of major alleles for the four SNPs, demonstrated substantially higher RLEs

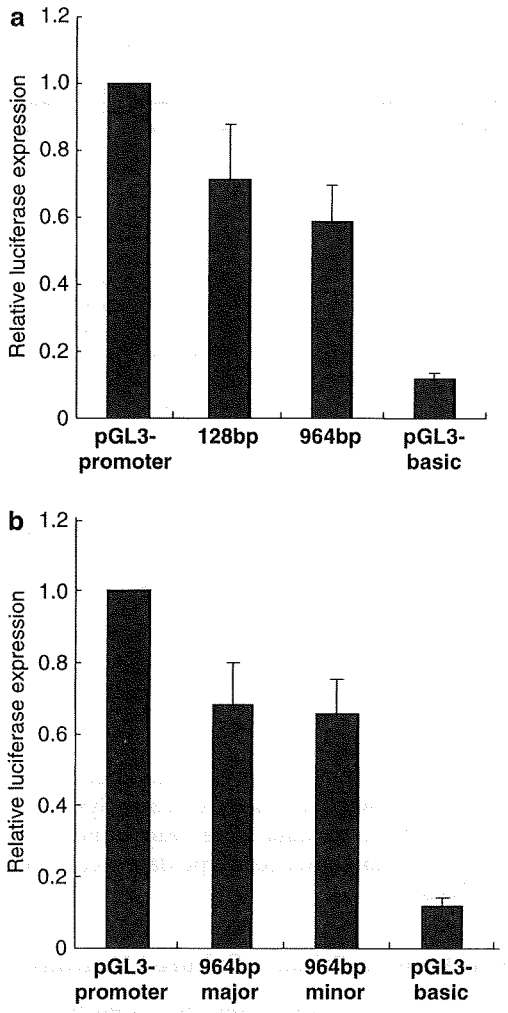


Figure 2 Promoter assay. (a) RLE for pGL3-Basic vector with insertion of 128 and 964 bp of the hNP 5′ flanking regions in comparison with pGL3-Basic vector, which dose not contain a promoter sequence. The RLE for pGL3-promoter vector containing SV40 promoter (positive control vector) was assigned a value of 1. Both 128- and 964-bp fragments showed substantially higher RLE compared with pGL3-basic vector without promoter sequence. (b) Comparison of RLE between the major (G-C-A-G for SNPs 1-2-3-4) and minor (T-T-A-C) alleles. No significant difference was found between the two alleles.

(relative luciferase expression) than that of pGL3-basic empty vector, suggesting that the core promoter region is located within the 128-bp fragment. We then cloned the 964-bp allele-specific promoter fragments (SNP1-2-3-4; major allele: G-C-A-G, minor allele: T-T-A-C) and compared RLEs between the two alleles (Figure 2b); however, we found no significant difference in the RLE between the two haplotype fragments. These results suggest that SNPs 1, 2, and 4 might not influence the transcriptional activity of the hNP gene.

Association with Psychiatric Diseases

We genotyped five SNPs (SNPs 3, 4, 6, 7, and 23) to examine possible association with schizophrenia, major depression, and bipolar disorder. Genotype and allele distributions in the diagnostic groups are shown in Table 1. Genotype

Table 1 Genotype and Allele Distributions of the five SNPs of the hNP Gene in Patients with Schizophrenia, those with Major Depression, those with Bipolar Disorder, and the Controls

SNP	Diagnosis	N	Genotype frequency (GF)			Allele frequency (AF)		Odds ratio (95% CI)	χ^2-Test *vs* controls		
									GF *vs* HW	GF (df = 2)	AF (df = 1)
SNP3			A/A	A/G	G/G	A	G				
	Controls	696	462 (0.66)	208 (0.30)	26 (0.04)	1132 (0.81)	260 (0.19)		0.67		
	SZ	421	277 (0.66)	126 (0.30)	18 (0.04)	680 (0.81)	162 (0.19)	1.06 (0.85–1.33)	0.45	$\chi^2 = 0.21$ $P = 0.90$	$\chi^2 = 0.11$ $P = 0.74$
	MD	382	276 (0.72)	90 (0.24)	16 (0.04)	642 (0.84)	122 (0.16)	1.18 (0.93–1.51)	0.02	$\chi^2 = 4.94$ $P = 0.08$	$\chi^2 = 2.48$ $P = 0.12$
	BD	202	139 (0.69)	56 (0.28)	7 (0.03)	334 (0.83)	70 (0.17)	1.09 (0.82–1.46)	0.65	$\chi^2 = 0.4$ $P = 0.81$	$\chi^2 = 0.36$ $P = 0.54$
SNP4			G/G	G/C	C/C	G	C				
	Controls	683	388 (0.57)	243 (0.36)	52 (0.08)	1019 (0.75)	347 (0.25)		0.11		
	SZ	406	234 (0.58)	150 (0.37)	22 (0.05)	618 (0.76)	194 (0.24)	1.1 (0.90–1.36)	0.75	$\chi^2 = 1.97$ $P = 0.37$	$\chi^2 = 0.62$ $P = 0.43$
	MD	371	219 (0.59)	126 (0.34)	26 (0.07)	564 (0.76)	178 (0.24)	1.1 (0.89–1.36)	0.19	$\chi^2 = 0.5$ $P = 0.78$	$\chi^2 = 0.58$ $P = 0.47$
	BD	198	91 (0.46)	90 (0.45)	17 (0.09)	272 (0.69)	124 (0.31)	1.33 (1.04–1.7)	0.43	$\chi^2 = 7.47$ $P = 0.023$	$\chi^2 = 5.35$ $P = 0.012$
SNP6			T/T	T/A	A/A	T	A				
	Controls	711	316 (0.44)	306 (0.43)	89 (0.13)	938 (0.66)	484 (0.34)		0.27		
	SZ	422	195 (0.46)	192 (0.45)	35 (0.08)	582 (0.69)	262 (0.31)	1.17 (0.97–1.41)	0.20	$\chi^2 = 4.86$ $P = 0.09$	$\chi^2 = 2.15$ $P = 0.14$
	MD	378	171 (0.45)	164 (0.43)	43 (0.11)	506 (0.67)	250 (0.33)	1.06 (0.88–1.29)	0.70	$\chi^2 = 0.31$ $P = 0.86$	$\chi^2 = 0.21$ $P = 0.65$
	BD	197	70 (0.36)	99 (0.50)	28 (0.14)	239 (0.61)	155 (0.39)	1.25 (0.99–1.58)	0.46	$\chi^2 = 5.03$ $P = 0.08$	$\chi^2 = 3.8$ $P = 0.051$
SNP7			A/A	A/G	G/G	A	G				
	Controls	718	325 (0.45)	314 (0.44)	79 (0.11)	964 (0.67)	472 (0.33)		0.81		
	SZ	433	209 (0.48)	190 (0.44)	34 (0.08)	608 (0.70)	258 (0.30)	1.16 (0.96–1.40)	0.31	$\chi^2 = 3.26$ $P = 0.20$	$\chi^2 = 2.36$ $P = 0.12$
	MD	387	182 (0.47)	163 (0.42)	42 (0.11)	527 (0.68)	247 (0.32)	1.05 (0.87–1.28)	0.55	$\chi^2 = 0.33$ $P = 0.85$	$\chi^2 = 0.21$ $P = 0.65$
	BD	203	72 (0.35)	103 (0.51)	28 (0.14)	247 (0.61)	159 (0.39)	1.31 (1.04–1.65)	0.36	$\chi^2 = 6.2$ $P = 0.042$	$\chi^2 = 5.56$ $P = 0.018$
SNP23			A/A	A/G	G/G	A	G				
	Controls	714	428 (0.60)	241 (0.34)	45 (0.06)	1097 (0.77)	331 (0.23)		0.16		
	SZ	421	267 (0.63)	135 (0.32)	19 (0.05)	669 (0.79)	173 (0.21)	1.17 (0.94–1.44)	0.71	$\chi^2 = 2.25$ $P = 0.32$	$\chi^2 = 2.13$ $P = 0.14$
	MD	388	240 (0.62)	127 (0.33)	21 (0.05)	607 (0.78)	169 (0.22)	1.08 (0.87–1.34)	0.44	$\chi^2 = 0.56$ $P = 0.75$	$\chi^2 = 0.56$ $P = 0.45$
	BD	204	98 (0.48)	86 (0.42)	20 (0.10)	282 (0.69)	126 (0.31)	1.48 (1.16–1.88)	0.86	$\chi^2 = 9.82$ $P = 0.0073$	$\chi^2 = 10.07$ $P = 0.0015$

Abbreviations: 95% CI, 95% confidence interval; BD, bipolar disorder; df, degrees of freedom; hNP, human neuropsin; HW, Hardy–Weinberg; MD, major depression; SNP, single-nucleotide polymorphism; SZ, schizophrenia.Significant *p*-values are gray colored.

distributions of these SNPs did not deviate significantly from Hardy–Weinberg equilibrium, except for SNP3 in patients with major depression ($P = 0.02$). There was no significant difference in genotype or allele distribution for any SNP between patients and controls for schizophrenia or major depression. However, there was a significant difference in genotype distributions between patients with bipolar disorder and controls for three SNPs, that is, SNPs 4, 7, and 23. Allele frequencies for these SNPs also differed significantly between the two groups. *P*-values, odds ratios, and their 95% confidence interval (CI) are shown in Table 1. Then we performed haplotype-based analysis with a two-marker sliding window method. We obtained no evidence of a significant association for schizophrenia or major depression (data not shown). With respect to bipolar disorder, we obtained significant individual *P*-values for all combinations of two markers; however, significant global *P*-value (0.0068) was obtained only when haplotype consisted of SNPs 7 and 23 (Table 2). Furthermore, overall global *P*-value ($P = 0.083$), considering all multiple testing for all the combinations of two-marker haplotypes, just failed to reach statistical significance. Thus, we did not obtain any stronger evidence for association in the haplotype-based analysis than in the single-marker analysis of SNP23 ($P = 0.0015$).

Association with Memory and IQ

Among the 166 controls whose memory scale and IQ were measured, SNP23 (A/G) was successfully genotyped in 163 individuals. Mean (SD) index scores of verbal memory, visual memory, general memory, attention and concentration, and delayed recall in the 163 controls were 110.9 (13.7), 109.9 (9.0), 112.2 (12.1), 103.9 (13.5), and 112.1

Table 2 Two-Marker Haplotype Analysis in Patients with Bipolar Disorder and Controls.

Markers					Haplotype frequency			P-value	
SNP3	SNP4	SNP6	SNP7	SNP23	BD	Controls	Individual	Global[a]	Overall global[a]
A	C				0.31	0.26	0.028	0.073	
	C	A			0.30	0.24	0.030	0.10	
		A	G		0.39	0.33	0.028	0.11	
			G	G	0.30	0.23	0.0068	0.014	0.083

Abbreviations: BD, bipolar disorder; SNP, single-nucleotide polymorphism.
[a]Global *P*-value for each combination of two markers and overall global significance for all combinations of two markers were calculated by permutation of 10 000 simulations.

(12.0), respectively. Mean (SD) full-scale IQ, verbal IQ, and performance IQ were 109.3 (11.6), 107.3 (12.9), and 110.3 (11.7), respectively. Since SNP23 showed the strongest association with bipolar disorder (G-allele was the risk allele) among the 5 SNPs examined, memory and IQ were compared between those who carried the G-allele (carrier, G/G or A/G, $N=64$) and those who did not (non-carrier, A/A, $N=99$) (Figure 3). Since the number of individuals with G/G genotype was very small ($N=8$), they were combined with those with the A/G genotype. With respect to sub-scales of WMS-R, the mean score of attention/concentration was significantly lower in carriers than in non-carriers ($P=0.016$); however, there were no significant differences between the two groups for the remaining sub-scales (verbal memory, visual memory, general memory, and delayed recall). With respect to WAIS-R, there was a significant difference in full-scale IQ ($P=0.018$) between the two groups. When verbal and performance IQ were examined separately, there was a highly significant difference in verbal IQ ($P<0.001$), but not in performance IQ, between the two groups. The mean verbal IQ (SD) for carriers and non-carriers was 103.4 (12.9) and 109.8 (12.3), respectively. As for the other SNPs, similar results are obtained (data not shown) because of the tight LD across the SNPs.

DISCUSSION

In the present study, we performed polymorphism screening and identified 28 SNPs, including nine novel SNPs, in the 7428-bp region of the whole hNP gene, including the 5′ and 3′ flanking regions. Then we performed promoter assay and determined a core promoter region of the hNP gene, although failing to find significant effects of SNPs on transcriptional activity. Association analysis using five SNPs as markers revealed significant difference in genotype and allele distributions for some of the SNPs between patients and controls for bipolar disorder, but not for schizophrenia or major depression. When a possible association of the SNPs with memory and IQ was examined in healthy control subjects, we found significant differences in attention/concentration sub-scale score of the WMS-R and verbal IQ between genotypes.

Among the 28 SNPs identified, there was only one SNP in the exons; SNP22 was a Val286Ile missense mutation in exon 6, which was detected in a patient with schizophrenia. Additional genotyping for 178 schizophrenic subjects did not find anyone carrying this variant, indicating that this is a rare mutation. Thus, whether this mutation is pathogenic or not is unclear. Since we examined only 24 individuals for polymorphism screening, we may have missed some rare mutations as the SNP22.

Our promoter assay in rat primary cultured neurons suggested that the core promoter is present in the 128-bp 5′ upstream region of the hNP gene. Since the RLE of pGL3-vector containing the 964-bp fragment was somewhat lower than that of pGL3-vector containing 128-bp fragment, a silencer-like region may be present between 128- and 964-bp positions upstream of the hNP gene. Then we examined whether transcriptional activity differs in an allele-dependent manner; however, we found no significant difference between alleles. These results suggest that SNPs 1, 2, and 4 might not influence the transcriptional activity of the hNP gene. According to the TFSEARCH database (http://mbs.cbrc.jp/research/db/TFSEARCHJ.html), these SNPs are not located on any of the binding sites of transcriptional factors, which is in line with our finding of no significant difference between the alleles.

In our association study with psychiatric diseases, we found, for the first time, significant differences in genotype and allele distributions between patients with bipolar disorder and controls. The best *P*-value was obtained for SNP23 in allele distribution ($P=0.0015$, odds ratio 1.48, 95% CI 1.16–1.88). This *P*-value remained significant even after correcting the critical *P*-value for Bonferroni's multiple testing (15 comparisons: 5 SNPs × 3 diseases). Haplotype-based analysis also yielded nominally significant results particularly when SNP23 was included in markers of analysis. These results suggest that SNP23 or other unknown SNPs in LD with SNP23 confers susceptibility to bipolar disorder. Since hNP is a part of a gene cluster (kallikreins), there remains a possibility that variations of some other kallikrein gene might be truly responsible to giving susceptibility to bipolar disorder. The results are in line with a previous study reporting a susceptibility locus for bipolar disorder on chromosome 19q13 (Badenhop *et al*, 2002). A possible limitation is that a portion of patients with bipolar disorder and controls were recruited in a geographically different area (ie, Shiga prefecture but not in Tokyo), which may have resulted in a population stratification; however, the minor allele frequency of SNP23 was very similar in controls from Shiga and those from Tokyo (0.233 in Shiga and 0.232 in Tokyo), suggesting that the effect of stratification is unlikely. Another limitation might be that

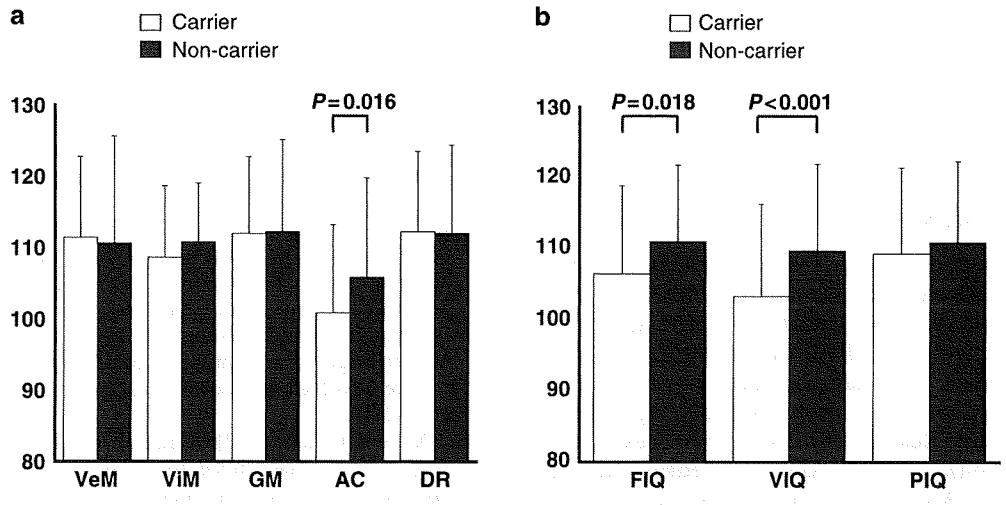


Figure 3 Relationship of memory and IQ with genotype of SNP23. Memory and IQ were compared between those who carried the G allele (carrier: G/G or A/G) of SNP23 and those who did not (non-carrier: A/A). (a) Memory and genotype. VeM, verbal memory; ViM, visual memory; GM, general memory; AC, attention and concentration; DR, delayed recall. (b) IQ and genotype. FIQ, full-scale IQ; VIQ, verbal IQ; PIQ, performance IQ.

we did not conduct structured interview for diagnosis of the patients. However, consensus diagnosis was made by at least two psychiatrists one of whom was in charge of the patients; thus, the possibility of misdiagnosis might be minimal. In addition, our sample size (207 bipolar disorder subjects and 727 controls) was not very large, and thus further investigations in other samples are required to draw any conclusion. With respect to schizophrenia or major depression, we did not obtain any evidence for association with hNP.

Interestingly, we found significant association of memory and IQ with the hNP gene in healthy subjects. Carrying the G-allele of SNP23, the risk allele for bipolar disorder, was associated with lower score in attention/concentration assessed with the WMS-R ($P=0.016$) and lower verbal IQ assessed with WAIS-R ($P<0.001$). The evidence for the former association (with attention/concentration) was weak and it would not be significant any more after correcting for multiple testing; however, the latter association (with verbal IQ) was highly significant and remained significant even when multiple testing was taken into consideration. Since bipolar disorder shows a wide range of cognitive deficits, including memory and IQ (Schretlen *et al*, 2007; Daban *et al*, 2006), the observed impact on intelligence may have some relevance to susceptibility to bipolar disorder. However, given that deficits in intelligence and memory are generally worse in schizophrenia than in bipolar disorder, alterations in hNP may have some effects specific to molecular mechanisms of bipolar disorder.

NP is a secretory serine protease that degrades cell adhesion molecule L1 (CAM-L1) (Matsumoto-Miyai *et al*, 2003) and is possibly involved in the synaptogenesis and maturation of orphan and small synapses (Nakamura *et al*, 2006). Furthermore, NP has been shown to be involved in activity-dependent synaptic plasticity, that is, LTP and kindling epileptogenesis (Komai *et al*, 2000; Okabe *et al*, 1996). As mentioned above, the type 2 splice variant has been shown to be expressed as abundant as the type 1 in human brain (Mitsui *et al*, 1999) and the hominoid-specific form (Li *et al*, 2004), which occurred through a human-specific T-to-A mutation (c.71–127T>A) during primate evolution (Lu *et al*, 2007). Taken together, NP is involved in synaptic plasticity via modulation of synaptic structure, and may play an important role in brain function of higher order such as learning, memory, and mental disorders. With respect to psychiatric diseases, indeed, altered expression levels of CAM-L1 mRNA and protein have been reported in postmortem brains of depressed patients (Laifenfeld *et al*, 2005). In line with this, chronic antidepressants increase expression levels of CAM-LI in rats (Sairanen *et al*, 2007; Laifenfeld *et al*, 2002). It would be intriguing to examine the expression levels of NP in postmortem brains of psychiatric patients.

We found that SNP23 is most associated with bipolar disorder among the examined SNPs. Haplotype-based analysis did not yield any stronger results, suggesting that SNP23 may be responsible for giving susceptibility to bipolar disorder. In addition, SNP23 showed strong impact on verbal IQ in healthy subjects. SNP23 is located 69 bp downstream to the 3′ end of exon 6 (the final exon). Thus SNP23 is on the 3′ regulatory region of the hNP gene. Growing evidence has shown that 3′ regulatory regions of human genes play an important role in regulating mRNA 3′ end formation, stability/degradation, nuclear export, subcellular localization, and translation, and are consequently rich in regulatory elements. Indeed, several diseases have been reported to be associated with variants in the 3′ regulatory region (Chen *et al*, 2006). Notably, the major allele (A) of SNP23 differs from the corresponding base (G) in monkeys or apes (ie, rhesus macaques or chimpanzees), according to the UCSC database, and thus it is not evolutionally conserved. It is interesting that carriers of the G allele were found to be poorer in memory and IQ subscales than individuals with A/A genotype in the present study. Although SNP23 is not located on obvious motifs or conserved sequence elements, it is also possible that this human-specific mutation may contribute to the higher memory and intelligence functions in humans. If our results

are replicated in other samples, it is important to elucidate the possible functional effects of SNP23 on regulation of hNP mRNA, which may contribute to understanding of the pathogenesis of bipolar disorder and brain function of higher order specific to human beings.

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DISCLOSURE/CONFLICT OF INTEREST

All authors declare that they have no conflict of interests to disclose.

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Glucocorticoid Prevents Brain-Derived Neurotrophic Factor-Mediated Maturation of Synaptic Function in Developing Hippocampal Neurons through Reduction in the Activity of Mitogen-Activated Protein Kinase

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An increased level of glucocorticoid may be related to the pathophysiology of depressive disorder. The involvement of brain-derived neurotrophic factor (BDNF) in the antidepressive effect has also been suggested; however, the possible influence of glucocorticoid on the action of BDNF in the developing central nervous system has not been elucidated. In this study, we investigated the effect of glucocorticoid (dexamethasone, DEX) on synaptic maturation and function enhanced by BDNF in early developing hippocampal neurons. In the immature stage, BDNF increased the outgrowth of dendrites and the expression of synaptic proteins including glutamate receptors and presynaptic proteins. Pretreatment with DEX significantly inhibited the BDNF-dependent up-regulation of both dendritic outgrowth and synaptic proteins. In the more mature stage, the BDNF-reinforced postsynaptic Ca^{2+} influx was decreased by DEX. BDNF-enhanced presynaptic glutamate release was also suppressed. RU486, a glucocorticoid receptor antagonist, canceled the DEX-dependent blocking effect on the action of BDNF. After down-regulation of glucocorticoid receptor by small interfering RNA application, no inhibitory effect of DEX on the BDNF-increased synaptic proteins was observed. Interestingly, the BDNF-activated MAPK/ERK pathway, which is an essential intracellular signaling pathway for the BDNF-increased synaptic proteins, was reduced by DEX. These results suggest that BDNF-mediated synaptic maturation is disturbed after neurons are exposed to high-level glucocorticoid in their development stage. (*Molecular Endocrinology* 22: 546–558, 2008)

GLUCOCORTICOID IS A STRESS hormone that is regulated by the hypothalamic-pituitary-adrenal axis including the hippocampus (1). This hormone is needed to cope with stressful life events and regulates the expression of many target genes at the cellular level (2). However, failure in the control of glucocorticoid homeostasis is thought to be closely related to the symptoms of depressive disorder (3). We previously reported that acutely elevated glucocorticoid levels did not return to the basal level in depressive patients (4). Interestingly, increased glucocorticoid is reported to influence learning and memory in humans (5). Depressive patients' deficits in cognition, learning, and memory have also been reviewed (6, 7). These studies suggest that sustained elevation of glucocorticoid is involved in the pathophysiology of depression.

Many studies using *in vivo* and *in vitro* systems in animals have reported that excessive glucocorticoid causes neuronal damage. Cognitive or learning deficits in glucocorticoid-administered animals occur (8, 9). As expected, chronic stress and administration of glucocorticoid result in dendritic atrophy of hippocampal neurons (10–13). In medial prefrontal cortex, the dendrite atrophy induced by daily 10-min restraint stress for 1 wk was reported (14). In addition, infants that received maternal separation stress during postnatal d 2–14 (P2–P14) exhibited decreased mossy fiber in the hippocampus (15). On the other hand, an acute function of the glucocorticoid receptor (GR) is also shown, which appeared within a few minutes. GR

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Abbreviations: BDNF, Brain-derived neurotrophic factor; CNS, central nervous system; DEX, dexamethasone; DIV1, 1 d *in vitro*; DMSO, dimethylsulfoxide; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GluR1, glutamate receptor 1; GR, glucocorticoid receptor; KRH, HEPES-buffered Krebs Ringer assay buffer; MAP2, microtubule-associated protein 2; MR, mineralocorticoid receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMDA, *N*-methyl-D-aspartate; NR2A, NMDA receptor 2A; P2, postnatal d 2; pERK1/2, phosphorylated ERK1/2; siRNA, small interfering RNA; SNAP25, synaptosome-associated protein 25; TUJ1, class III β-tubulin.

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prolongs an acute *N*-methyl-D-aspartate receptor (NMDA receptor)-mediated increase in intracellular Ca^{2+} in hippocampal neurons (16). GR function is involved in a potentiation of the response to NMDA in dopamine-sensitive neurons in the ventral tegmental area (17). Taking these findings together, clarifying the action of glucocorticoid/GR signaling is important for understanding the symptoms of depressive disorder.

Recent reports suggest that the action of growth factors including neurotrophin is involved in depressive disorder. In particular, the decrease in brain-derived neurotrophic factor (BDNF) may be related to the pathophysiology of mental disorders. For example, BDNF expression is low in the brains of suicide victims with depressive disorder (18). A reduction in the concentration of serum BDNF was observed in depressive patients (19). In animal studies, the down-regulation of BDNF in the hippocampus of glucocorticoid-administered or stressed rats was confirmed (20, 21). Thus, down-regulation of BDNF expression may result in depressive disorder. As further evidence, the mechanism by which antidepressants exert the antidepressive effect also supports the importance of BDNF. The increase of BDNF level is thought to be a mechanism by which the antidepressive effect is exerted. Chronic treatment with antidepressants up-regulates the mRNA of BDNF in rats (22). We recently reported that antidepressants potentiated the BDNF function; that is, antidepressants reinforce BDNF-stimulated glutamate release via enhancing phospholipase $C\gamma/Ca^{2+}$ (PLCγ) intracellular signaling (23), suggesting that the reinforcement of BDNF-stimulated intracellular signaling is also involved in the antidepressive effect.

BDNF is a neurotrophic factor that promotes neuronal differentiation, survival, and plasticity in the peripheral nervous system and central nervous system (CNS) (24). In CNS, BDNF elicits long-term potentiation, which is related to synaptic plasticity (25). We previously reported that BDNF enhanced depolarization-induced release of glutamate (26) and potentiated the spontaneous Ca^{2+} oscillations in cultured cortical neurons (27), suggesting that BDNF strengthens the activity of neural networks. In developing CNS neurons, BDNF promotes neurite outgrowth (28) and increases the synaptic proteins including synapsin I, synaptophysin, and synaptotagmin (26, 29). Therefore, BDNF plays an important role in establishing synaptic connections and neuronal maturation.

Glucocorticoid is also reported to influence maturation of the CNS in animal experiments (30–33); however, the cellular mechanism underlying glucocorticoid action in neuronal development is largely unknown. Therefore, we focused on the cross-talk between BDNF and the glucocorticoid action *in vitro* system. There are two receptors for glucocorticoid: GR and mineralocorticoid receptor (MR) (34). GR is suggested to be related to the stress response because it has a lower affinity for glucocorticoid than MR. In the present study, we used dexamethasone (DEX), which is a selective agonist for GR (35), as an excessive glucocorticoid exposure model. We found that BDNF-increased neurite outgrowth and synaptic proteins in developing hippocampal neurons were suppressed by pretreatment with DEX. The down-regulation of BDNF-increased pre- and postsynaptic function was still observed in the more mature stage. Interestingly, DEX inhibited the BDNF-activated MAPK signaling, a required signaling for the BDNF-increased synaptic proteins and functions. Our present findings demonstrate that BDNF-reinforced synaptic maturation is disturbed in neurons by exposure to high-level glucocorticoid in their development stage.

RESULTS

BDNF-Enhanced Outgrowth of Neurites Was Suppressed by DEX Pretreatment

To investigate the influence of glucocorticoid (DEX) on the maturation of synaptic connections and functions, in which BDNF exerts important effects, we first focused on the change of structure of young hippocampal neurons. In our cultures, the glutamic acid decarboxylase (GAD)-positive γ-aminobutyric acid (GABA)-ergic neurons were about 10% (Fig. 1A, *yellow* in b); thus, the other microtubule-associated protein 2 (MAP2)-positive cells are expected to be glutamatergic neurons (Fig. 1A, a and b). As illustrated in Fig. 1B, DEX (final 10 μM) was applied at 1 d *in vitro* (DIV1) before the addition of BDNF (100 ng/ml, at DIV2). Forty-eight hours (at DIV4) after BDNF addition, the neurite outgrowth was determined. As shown in Fig. 1C, BDNF tends to enhance neurite outgrowth of GABAergic (MAP2/GAD-double-positive cells) neurons (Fig. 1C, a–c and d–f), and pretreatment with DEX slightly inhibited the effect of BDNF (Fig. 1C, d–f and g–i), although significance was not observed. Sole DEX treatment had no effects (Fig. 1C, a–c and j–l). The thickness of neurites seemed to be increased by BDNF; however, the number of primary neurites per cell for each experimental condition revealed no significant change (Fig. 1C, m). Next, the effect of DEX on glutamatergic neurons was examined. BDNF increased the number of neurites of glutamatergic (MAP2/glutamate-double-positive) neurons (Fig. 1D, a–c and d–f). Interestingly, DEX pretreatment blocked the BDNF-enhanced outgrowth (Fig. 1D, d–f and g–i). Sole DEX treatment exerted no influence (Fig. 1D, a–c and j–l). Quantitative data show the inhibitory effect of DEX on the BDNF-increased glutamatergic neurites (Fig. 1D, m). The number of neurites of the MAP2-positive but GAD-negative neurons, most of which were considered to be glutamatergic neurons, was also examined [none, 4.08 $\pm$ 1.44 (n = 76 cells); BDNF, 7.46 $\pm$ 2.84 (n = 82); BDNF plus DEX, 4.64 $\pm$ 2.20 (n = 105); DEX, 3.54 $\pm$ 1.38 (n = 97)] and confirmed the inhibitory effect of DEX. We confirmed that treatment with DEX and/or BDNF did not affect cell survival at DIV4 (supplemental Fig. 1, published as

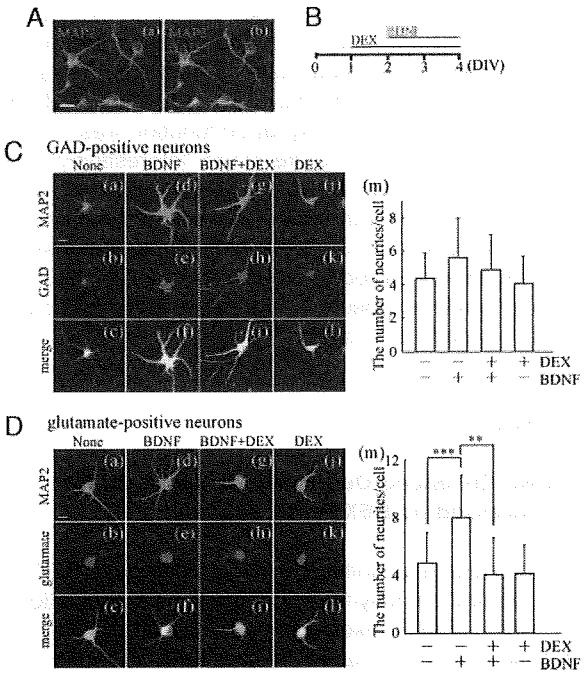

Fig. 1. BDNF-Enhanced Outgrowth of Neurites Was Suppressed in the Presence of DEX

A, Cultured hippocampal neurons at DIV4 were immunostained with anti-MAP2 (*green*, neuronal dendritic marker) and anti-GAD (*red*, GABAergic neuronal marker) antibodies. a, MAP2 image; b, MAP2 and GAD merged image. Approximately 10% of MAP2-positive cells were GAD-positive GABAergic neurons in our cultures. *Bar*, 20 μm. B, Experimental schedule of BDNF and DEX application for immunostaining. DEX was applied at DIV1, and then BDNF was also applied at DIV2 in the presence of DEX. Forty-eight hours after BDNF addition, the cultured cells were fixed for immunostaining. C, Influence of DEX treatment on neurite outgrowth of GABAergic neurons. a, d, g, and j, The neurites were revealed by immunostaining with anti-MAP2 antibody; b, e, h, and k, GABAergic neurons were determined by staining with anti-GAD antibody; c, f, i, and l, merged images; d–f, BDNF has a slight tendency to increase the number of neurites compared with control (a–c) (none), although significance was not observed; g–i, DEX pretreatment before BDNF application; j–l, sole DEX treatment. *Bar*, 20 μm. m, The number of neurites per cell was counted. Data represent mean ± SD (none, n = 60; BDNF, n = 58; BDNF plus DEX, n = 73; DEX, n = 48). The n indicates the number of cells. D, Effect of DEX on neurite outgrowth of glutamatergic neurons. MAP2-positive (a, d, g, and j) and glutamate-positive (b, e, h, and k) glutamatergic neurons are shown. c, f, i, and l, Merged images; d–f, BDNF increased the number of neurites compared with control (a–c); g–i, DEX significantly suppressed the BDNF effect; j–l, sole DEX had no influence compared with control. *Bar*, 20 μm. m, Quantification indicates that the number of glutamatergic neurites was increased by BDNF, and the increase was suppressed by DEX. Data represent mean ± SD (none, n = 200; BDNF, n = 200; BDNF plus DEX, n = 200; DEX, n = 200). The n indicates the number of cells. Reproducibility was confirmed by three independent cultures. Statistical significance was determined by ANOVA. ***, $P < 0.001$; **, $P < 0.01$.

supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). These results suggest that DEX suppresses the BDNF-enhanced neurite outgrowth in early developing hippocampal neurons.

BDNF Did Not Increase Expression of Synaptic Proteins in the Presence of DEX

The change of neurite outgrowth might be associated with synaptic connection. Thus, expressions of pre- and postsynaptic proteins were examined. As expected, the application of BDNF dramatically increased various synaptic proteins including NMDA receptor 2A (NR2A), NR2B, glutamate receptor 1 (GluR1), synapsin I, and synaptosome-associated protein 25 (SNAP25). DEX pretreatment (final 0.1–100 μM) inhibited the effect of BDNF in a dose-dependent manner (Fig. 2A). Quantification after Western blotting was performed (Fig. 2B). The expression of postsynaptic proteins (glutamate receptor subunits), such as

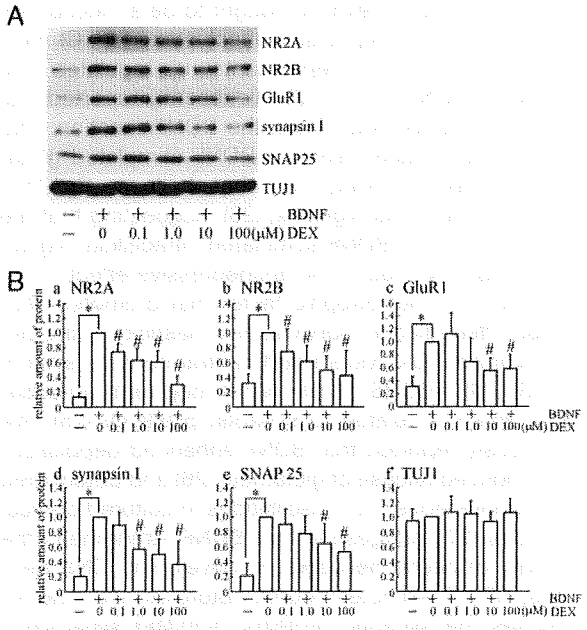

Fig. 2. BDNF-Increased Expression of Synaptic Proteins Was Inhibited by Pretreatment with DEX

A, Dose-dependent effect of DEX on the BDNF-increased synaptic proteins. BDNF dramatically increased pre- and postsynaptic proteins at DIV4 (48 h after BDNF application, see legend to Fig. 1B). BDNF-increased synaptic proteins were inhibited by DEX in a dose-dependent manner. B, Quantitative analysis revealed that BDNF increased NR2A (a), NR2B (b), GluR1 (c), synapsin I (d), and SNAP25 (e). DEX pretreatment inhibited the BDNF effect. f, Expression of TUJ1 (class III β-tubulin), a neuronal marker, was not changed. Data represent mean ± SD (n = 6, from six independent cultures). Data were normalized to a level in BDNF stimulation without DEX pretreatment. Statistical significance was confirmed by Kruskal-Wallis test and Mann-Whitney *U* test. *, $P < 0.05$ (control *vs.* BDNF); #, $P < 0.05$ (BDNF *vs.* BDNF plus DEX).

NR2A (Fig. 2B, a), NR2B (Fig. 2B, b), and GluR1 (Fig. 2B, c), were up-regulated by BDNF. Presynaptic proteins, synapsin I (Fig. 2B, d), SNAP25 (Fig. 2B, e), were also increased by BDNF. The level of class III β-tubulin (TUJ1, a neuronal marker) is shown as a control (Fig. 2B, f). The increases of these synaptic proteins caused by BDNF were inhibited by DEX in a dose-dependent manner (Fig. 2B). As depicted in supplemental Fig. 2A (published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org), DEX application alone seemed to have a tendency to reduce the level of synaptic proteins compared with control; however, quantification performed with the result of DEX (10 μM) showed no significance (supplemental Fig. 2B). To quantify Western blotting, normalization using response to exogenous BDNF application was performed because control protein levels are unstable due to their low expression in immature neurons and because endogenous expression of BDNF remains unaltered in response to 72 h exposure of DEX (supplemental Fig. 2C).

DEX Decreased the BDNF-Enhanced Postsynaptic Function in More Mature Neurons

The inhibitory effect of DEX on the actions of BDNF in developing hippocampal neurons may result in a decrease in synaptic function of a more mature stage. Thus, to examine postsynaptic function, we performed Ca^{2+} imaging analysis at DIV7 (5 d after BDNF application). Fluo-3-filled cell images and time-course analysis of the BDNF-potentiated Ca^{2+} increase triggered by glutamate (final 1 μM) are shown (Fig. 3, A and B). Glutamate is expected to evoke the Ca^{2+} influx through ionotropic glutamate receptors. As shown, an increase in intracellular Ca^{2+} concentration stimulated by glutamate was potentiated by BDNF. However, DEX pretreatment (1 μM) reduced the BDNF-potentiated effect (Fig. 3, A and B). Quantification of the level of increased Ca^{2+} was conducted (Fig. 3C). Stimulation by high KCl (final 50 mM) was also tested. The BDNF-dependent enhancement in the Ca^{2+} increase stimulated by high KCl was blocked by DEX (Fig. 3D). Sole DEX treatment has an inhibitory effect compared with control, implying the involvement of influence of DEX on voltage-sensitive ion channels (Ca^{2+}, K^+ channels, *etc.*) in case of KCl stimulation. These results suggest that the inhibitory effect of DEX on BDNF-increased postsynaptic proteins in developing neurons results in a decrease in postsynaptic function of a more mature stage.

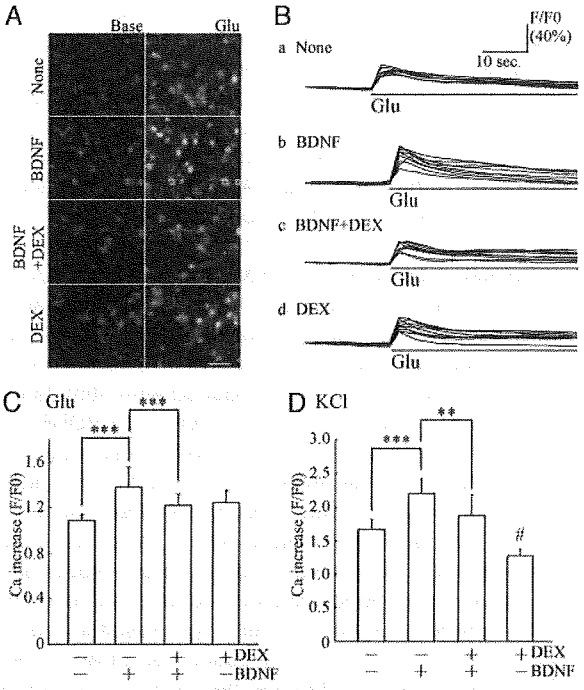

Fig. 3. DEX Decreased the BDNF-Potentiated Ca^{2+} Increase Triggered by Glutamate or High KCl Stimulation

A, Intracellular Ca^{2+} mobilization was monitored after Fluo-3 loading. *Left*, Basal intensity of fluorescence in Fluo-3-filled cells for each experimental condition; *right*, images after glutamate (final 1 μM) stimulation. These images were obtained 4 sec before (Base) and after glutamate stimulation (Glu), respectively. *Bar*, 50 μm. B, Time-course analysis of intracellular Ca^{2+} before and after glutamate addition. Each trace indicates the representative intensity of fluorescence from nine neurons in untreated cultures (none) (a) and cultures pretreated with BDNF (b), BDNF plus DEX (c), and DEX (d). *Bars* indicate exposure time to glutamate. Data are shown as a ratio (F/F0; intensity after stimulation/ basal intensity before stimulation). C, Quantitative analysis of Ca^{2+} increase induced by glutamate (1 μM). Enhancement of intracellular Ca^{2+} increase was observed in BDNF-treated cultures. Pretreatment of DEX (1 μM) inhibited the BDNF-potentiated Ca^{2+} increase. Data represent mean ± SD. Fluorescent intensity was measured from cells in a sister culture (n is the number of cells selected randomly): none, n = 33; BDNF, n = 46; BDNF plus DEX, n = 48; DEX, n = 49. ***, $P < 0.001$ (Kruskal-Wallis test and Mann-Whitney *U* test). The Ca^{2+} imaging analysis was performed at DIV7 (5 d after BDNF application). D, KCl-induced Ca^{2+} increase was also potentiated by BDNF and inhibited by DEX. High potassium (high KCl solution, final 50 mM) was applied to induce cell depolarization. Data represent mean ± SD. None, n = 9; BDNF, n = 13; BDNF plus DEX, n = 8; DEX, n = 11. ***, $P < 0.001$; **, $P < 0.01$; #, $P < 0.05$ (none *vs.* DEX), Kruskal-Wallis test and Mann-Whitney *U* test.

DEX Decreased the BDNF-Enhanced Presynaptic Function in More Mature Neurons

We then examined the presynaptic function in mature neurons after BDNF treatment with or without DEX pretreatment during the development stage. Because changes in the expression of presynaptic proteins occurred (see Fig. 2), first, the number of presynaptic sites was estimated. The presynaptic site of neurons at DIV16 was determined after long maintenance. DEX was applied at DIV1 before the addition of BDNF (DIV2). Two weeks (DIV16) after BDNF addition, the neurons were fixed for immunostaining. Representa-

tive images of immunostaining with anti-synapsin I antibody indicated that BDNF increased the number of synapsin I-positive presynaptic sites (Fig. 4A, a–d; b and d are magnified images). DEX inhibited the BDNF effect (Fig. 4A, c–e, f; d and f are magnified). A significant reduction was observed after sole DEX treatment compared with control (Fig. 4A, a, b, and g, h; b and h are magnified). Quantification indicated that the BDNF-dependent increase of presynaptic sites was completely abolished by DEX exposure (Fig. 4A, i). Next, to clarify the function of individual presynaptic sites, exocytosis imaging analysis with FM1-43 dye was carried out. The intensity of FM1-43 fluorescence in presynaptic terminals was reduced after stimulation with high KCl (Fig. 4B, a and b). Although BDNF enhanced the elimination of the FM-43 fluorescence (Fig. 4B, c), DEX had little effect on the BDNF-enhanced elimination, implying that DEX did not affect the function of individual presynaptic sites. Thus, we measured the amount of released glutamate in hippocampal cultures after depolarization because the decrease in the number of synaptic sites might result in reduction of the total amount of released neurotransmitter. The basal levels of glutamate (before KCl stimulation) are shown as *white bars* in Fig. 4C. KCl stimulation rapidly induced glutamate release, and BDNF potentiated the depolarization-evoked release (Fig. 4C, *black bars*). Markedly, DEX inhibited the BDNF-potentiated glutamate release in a dose-dependent manner. These results suggest that the inhibition of BDNF-potentiated presynaptic glutamate release in DEX-pretreated cultures reflects a decrease in the number of presynaptic sites.

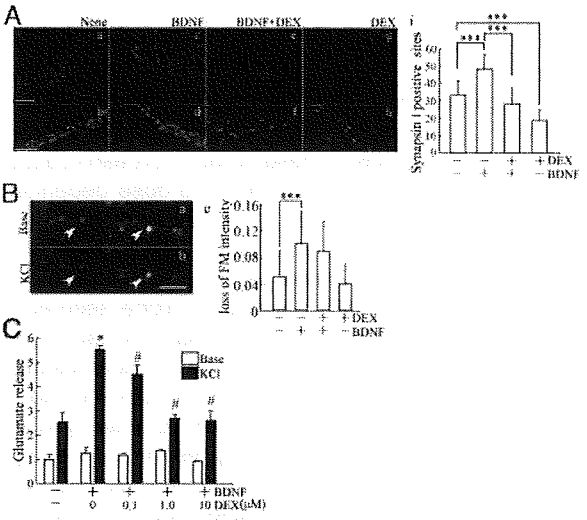

Fig. 4. BDNF Failed to Increase the Number of Presynaptic Sites and the Release of Glutamate after DEX Pretreatment

A, The number of presynaptic sites was quantified after immunostaining with anti-synapsin I antibody. *Top*, Representative images from untreated cultures (none) (a) and cultures pretreated with BDNF (c), BDNF plus DEX (e), and DEX (g). *Bar*, 20 μm. *Bottom*, High-magnification images of dendrites from untreated cells (none) (b) and cells pretreated with BDNF (d), BDNF plus DEX (f), and DEX (h). Bar, 10 μm. i, Quantification of the number of synapsin I-positive presynaptic sites per dendritic shaft (50 μm). Data represent mean ± SD. None, n = 39; BDNF, n = 40; BDNF plus DEX, n = 46; DEX, n = 42. The n is the number of dendritic shafts selected randomly from cultured neurons for each experimental condition. BDNF increased the synapsin I-positive presynaptic number, and DEX treatment canceled the BDNF effect. Neurons at DIV16 were fixed after long maintenance in the presence or absence of BDNF or DEX, respectively. ***, $P < 0.001$, ANOVA. B, Effect of DEX on BDNF-potentiated presynaptic exocytotic activity. FM-43 images of before (a) and after (b) KCl (50 mM) stimulation. FM-dye signal revealed the presynaptic sites (*arrowheads*). The exocytotic activity in presynaptic sites was determined by elimination of the fluorescence. c, Quantification of reduction of FM-dye fluorescence. BDNF enhanced the elimination of FM-dye fluorescence. DEX had no influence. Data represent mean ± SD. The n (= 30) is monitored fluorescence dots in neurons for each experimental condition. The presynaptic sites of DIV7 neurons were monitored. *Bar*, 5 μm. ***, $P < 0.001$, Kruskal-Wallis test and Mann-Whitney *U* test. C, Amount of glutamate released from hippocampal cultures. *White bars* indicate the basal release of glutamate without stimulation. *Black bars* show the glutamate release after KCl stimulation. The reduction caused by DEX (0.1–10 μM) in the BDNF-potentiated glutamate release was observed. The glutamate release in DIV7 cultures (5 d after BDNF application) was measured and indicated as relative release compared with basal release in control (without DEX and BDNF). Data represent mean ± SD (n = 4). The n indicates the number of wells for each experimental condition on a plate. *, $P < 0.05$ (control *vs.* BDNF); #, $P < 0.05$ (BDNF *vs.* BDNF plus DEX), Kruskal-Wallis test and Mann-Whitney *U* test.

DEX Showed No Inhibitory Effect in the Presence of GR Antagonist RU486

To investigate the mechanisms underlying the DEX-decreased action of BDNF, the possible involvement of GR was examined. Exposure of glucocorticoid is suggested to reduce GR protein expression (36). Consistently, application of DEX (10 μM) to hippocampal cultures at DIV1 prevented developmental increase of GR expression (Fig. 5A, a). On the other hand, MR expression was not changed (Fig. 5A, a). The down-regulation of GR after DEX addition was observed in a dose-dependent manner (Fig. 5A, b). In contrast, DEX did not affect the MR expression at any concentration of DEX (Fig. 5A, b). Quantification of GR expression after DEX exposure for 24 h was conducted (Fig. 5A, c). Furthermore, we tested the effect of RU486, an antagonist of GR. Levels of pre- and postsynaptic proteins including NR2A, NR2B, GluR1, synapsin I, and SNAP25 were examined (Fig. 5B, a). RU486 canceled the DEX-dependent decrease in BDNF-increased synaptic proteins. As a control, TUJ1 is also shown. The BDNF-enhanced Ca^{2+} increase was decreased in DEX-pretreated cultures; however, no reduction in Ca^{2+} increase was observed in coapplica-

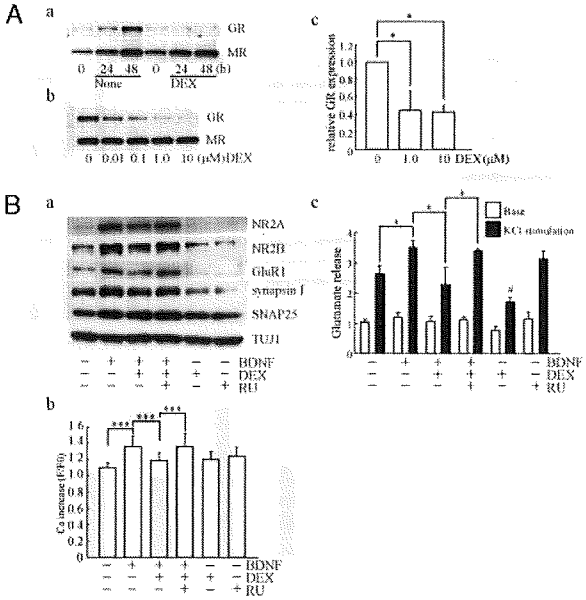

Fig. 5. Inhibitory Effect of DEX Was through GR

A, DEX inhibited increase in GR expression during *in vitro* maturation. a, GR and MR expression after DEX application at DIV2. No increase in GR expression was observed by DEX (10 μM) exposure for 24–48 h. MR expression was intact. b, DEX inhibited the increase in GR expression in a dose-dependent manner. DEX (0.01–10 μM) pretreatment was carried out at DIV1 for 24 h. MR expression was also shown. c, Quantitative analysis of GR expression 24 h after DEX (1.0 or 10 μM) application. Data represent mean ± SD; n = 4, from separate cultures. *, $P < 0.05$, Kruskal-Wallis test and Mann-Whitney *U* test. B, RU486, a GR antagonist, prevented DEX-dependent decrease in BDNF-dependent biological effects. a, RU486 (RU) reversed the DEX-dependent decrease in BDNF-increased synaptic proteins. Levels of pre- and postsynaptic proteins including NR2A, NR2B, GluR1, synapsin I, and SNAP25 are shown. As a control, TUJ1 is also detected. RU486 (1.0 μM) was applied 20 min before DEX (10 μM) addition. Twenty-four hours later, BDNF was added. b, RU486 prevented DEX-dependent decrease in BDNF-potentiated postsynaptic Ca^{2+} increase. RU486 (1.0 μM) was applied 20 min before DEX (1.0 μM) addition. Twenty-four hours later, BDNF was added. Imaging analysis was performed at DIV7 (5 d after BDNF application). Sole RU486 treatment had no effect. None, n = 57; BDNF, n = 147; BDNF plus DEX, n = 152; BDNF plus DEX plus RU486, n = 210; DEX only, n = 61; RU486 only, n = 133. Fluo-3 fluorescent intensity was measured from cells in a sister culture (n is the number of cells selected randomly). Data represent mean ± SD. ***, $P < 0.001$, ANOVA. c, RU486 reversed the DEX-dependent reduction in glutamate release stimulated by KCl. RU486, DEX, and BDNF were applied as described in b. Data represent mean ± SD; n = 4. The n indicates the number of wells for each experimental condition on a plate. *, $P < 0.05$; #, $P < 0.05$ (none *vs.* DEX), Kruskal-Wallis test and Mann-Whitney *U* test.

tion of DEX with RU486 (Fig. 5B, b). As expected, RU486 blocked the DEX-dependent decrease in the BDNF-enhanced glutamate release (Fig. 5B, c). Sole DEX treatment slightly reduced glutamate release, implying the possibility that a change in the activity of voltage-sensitive ion channels is involved.

To further clarify the involvement of GR, the effect of small interfering RNA (siRNA) for knockdown of GR was examined. The siRNA transfection was performed at DIV1. The levels of GR and MR at DIV2 indicated the GR-specific reduction after siRNA application (Fig. 6A, a–c). The expression levels of TUJ1 were not altered (Fig. 6A, a). After the GR knockdown by siRNA, DEX treatment before BDNF addition was carried out. As shown in Fig. 6B, DEX failed to decrease the BDNF-increased synaptic proteins after down-regulation of GR caused by siRNA. The levels of proteins at DIV5 are shown. DEX-dependent inhibition on the BDNF-increased NR2A, NR2B, GluR1, synapsin I, SNAP25, and synaptotagmin did not occur in GR-siRNA-transfected cultures, suggesting that GR function is involved in the inhibitory effect of DEX.

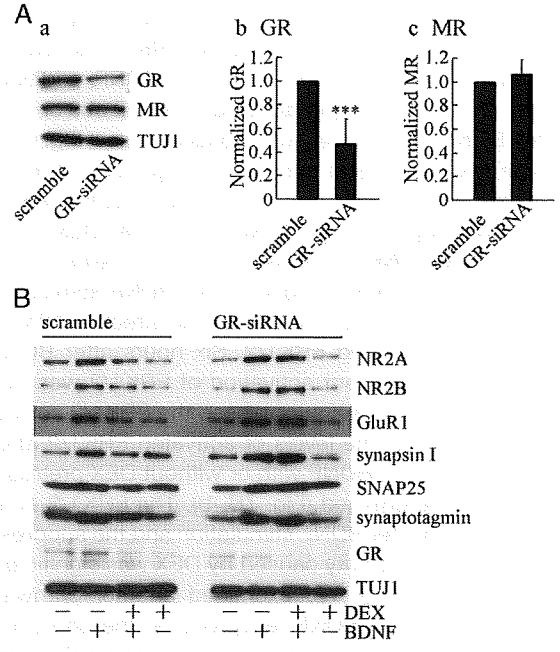

Fig. 6. DEX Did Not Exert Inhibitory Effect on the BDNF-Increased Synaptic Proteins after Down-Regulation of Endogenous GR by siRNA

A, a, The level of endogenous GR was decreased after GR-siRNA transfection. Scramble (control) siRNA had no effect. MR and TUJ1 are shown as a negative control. The levels of GR, MR, and TUJ1 were detected at DIV2. SiRNA transfection was performed at DIV1. b and c, Quantification of GR and MR expression at DIV2. Data represent mean ± SD; n = 4. Significant reduction was confirmed by Student's *t* test. ***, $P < 0.001$. B, DEX had no inhibitory effect on the BDNF-increased synaptic proteins after GR-siRNA transfection. The levels of proteins at DIV5 are shown. DEX-dependent inhibition on the BDNF-increased NR2A, NR2B, GluR1, synapsin I, SNAP25, and synaptotagmin did not occur in GR-siRNA-transfected cultures. SiRNA transfection was performed at DIV1, and then, DEX was applied at DIV2 followed by BDNF addition at DIV3. GR and TUJ1 were also shown.

DEX Decreased the BDNF-Increased Synaptic Proteins via Reducing Activation of the MAPK Pathway Stimulated by BDNF

To further determine what kind of mechanism is involved in DEX effects, we focused on the intracellular signaling. We previously reported that the activation of MAPK/ERK stimulated by BDNF is essential for BDNF-increased synaptic proteins in cultured cortical neurons (26). Thus, we tested the effect of the MAPK pathway inhibitor U0126 in the present system. U0126 (final 10 μM) was applied 20 min before adding BDNF at DIV2, and then samples were collected at DIV4 (Fig. 7A). U0126 suppressed the phosphorylation of ERK1/2 [activated ERK1/2 form, phosphorylated ERK1/2 (pERK1/2)]. The BDNF-dependent increase in the expression of synaptic proteins, including NR2A, NR2B, GluR1, synapsin I, and synaptotagmin, was prevented by U0126. Quantification of levels of pERK1/2 and synaptic proteins (48 h after BDNF application) was conducted (Tables 1 and 2, respectively). As shown in Table 1 and supplemental Fig. 3, we detected the BDNF-stimulated ERK1/2 activation at least 24 h, 48 h, and 5 d after BDNF addition, although the more highly activated ERK1/2 at 6 or 12 h after BDNF stimulation was observed (see Fig. 7B). These results suggest that the up-regulation of these synaptic proteins was through activation of the MAPK pathway triggered by BDNF. Because DEX suppressed the BDNF-increased synaptic proteins, it was possible that DEX inhibited the BDNF-stimulated ERK1/2. Thus, we next examined the effect of DEX on the BDNF-stimulated ERK1/2. Six hours after BDNF application, significant activation of ERK1/2 was observed; however, DEX suppressed the ERK1/2 activation (Fig. 7B, a). The levels of activated ERK1/2 after the application of BDNF with or without DEX pretreatment were determined (Fig. 7B, b). The ERK1/2 activation at 12 h after BDNF stimulation and the suppression of ERK1/2 activity caused by DEX was significant (Fig. 7B, c and d). We examined another pathway activated by TrkB (BDNF receptor), *i.e.* the phosphatidylinositol 3-kinase pathway. However, DEX pretreatment did not affect the activation of Akt (phosphorylated Akt, pAkt), a component of the phosphatidylinositol 3-kinase pathway, stimulated by BDNF (Fig. 7B, a and c). Total expression of Akt or ERK1/2 was not influenced by DEX (Fig. 7B, a and c). Finally, involvement of the MAPK pathway in BDNF-enhanced synaptic function was examined. U0126 completely blocked the BDNF-potentiated Ca^{2+} increase triggered by glutamate (Fig. 7C). The BDNF-enhanced glutamate release was also inhibited by U0126 (Fig. 7D). These results suggest that activation of the MAPK pathway is necessary for the BDNF-potentiated synaptic function and that the exposure to a high level of glucocorticoid suppressed the BDNF action through inhibition of the MAPK pathway.

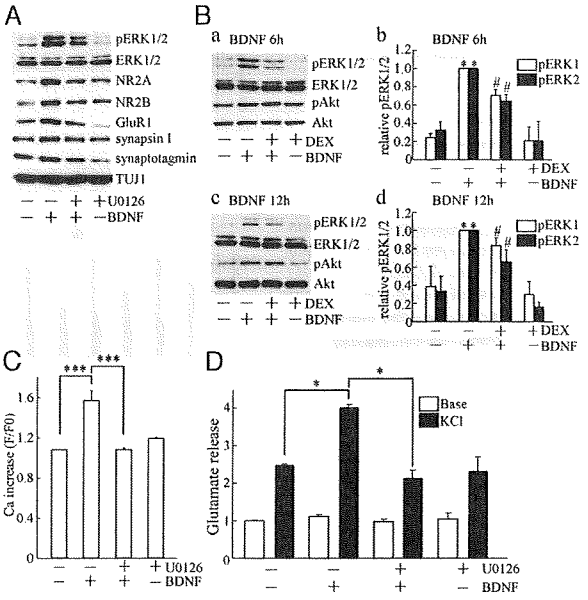

Fig. 7. Inhibitory Effect of DEX on the Action of BDNF Was via Reducing Activation of the MAPK Pathway

A, U0126 (a specific inhibitor of MAPK kinase, an upstream molecule of MAPK/ERK) suppressed the phosphorylation of ERK1/2 (pERK1/2) and the increases of synaptic proteins induced by BDNF. BDNF-up-regulated NR2A, NR2B, GluR1, synapsin I, and synaptotagmin was prevented by U0126. No change of total ERK1/2 was observed. U0126 (final 10 μM) was applied 20 min before BDNF addition. BDNF was added at DIV2. Forty-eight hours later, samples for immunoblotting were collected. B, BDNF-stimulated ERK activation was decreased by DEX (10 μM) pretreatment. a, Levels of pERK1/2, ERK1/2, pAkt, and Akt at 6 h after BDNF treatment are shown. DEX was applied at DIV1, and then BDNF was added at DIV2. The ERK1/2 activation was suppressed by DEX, whereas the Akt activation was intact. b, Quantitative analysis of activated ERK1/2 at 6 h was performed. Data represent mean ± SD; n = 4. *, $P < 0.05$ *vs.* basal pERK1/2 in untreated cells (without BDNF or DEX); #, $P < 0.05$ *vs.* BDNF-stimulated pERK1/2 without DEX pretreatment, Kruskal-Wallis test and Mann-Whitney *U* test. c, Activation of ERK1/2 and Akt at 12 h after BDNF application. The activated ERK1/2 by BDNF was still observed, and DEX inhibited the BDNF-activated ERK1/2. DEX did not affect the Akt activation by BDNF. d, Quantitative analysis of activated ERK1/2 at 12 h after BDNF application. Data represent mean ± SD; n = 4. *, $P < 0.05$ *vs.* basal pERK1/2 in untreated cells (without BDNF or DEX); #, $P < 0.05$ *vs.* BDNF-stimulated pERK1/2 without DEX pretreatment, Kruskal-Wallis test and Mann-Whitney *U* test. C, BDNF-potentiated Ca^{2+} increase required activation of the MAPK pathway. U0126 canceled the BDNF-potentiated Ca^{2+} increase. U0126 was applied 20 min before BDNF addition at DIV2. Ca^{2+} imaging was carried out at DIV7 (5 d after BDNF application). None, n = 50; BDNF, n = 50; BDNF plus DEX, n = 50; DEX, n = 50. The n indicates the number of cells. Data represent mean ± SD. ***, $P < 0.001$, ANOVA. D, BDNF-potentiated glutamate release was blocked by U0126. U0126 and BDNF were applied as indicated in C; n = 4. The n indicates the number of wells for each experimental condition on a plate. *, $P < 0.05$, Kruskal-Wallis test and Mann-Whitney *U* test.

Table 1. Effect of U0126 on the Activation of ERK1/2 Stimulated by BDNF

	None	BDNF	BDNF + U0126	U0126
pERK1	0.26 ± 0.14	1.00[a]	0.39 ± 0.32[b]	0.18 ± 0.15
pERK2	0.37 ± 0.21	1.00[a]	0.51 ± 0.27[b]	0.21 ± 0.16
ERK1	0.90 ± 0.12	1.00	1.13 ± 0.32	1.28 ± 0.47
ERK2	0.90 ± 0.12	1.00	0.97 ± 0.52	1.00 ± 0.18

Each band was quantified by densitometry after Western blot analysis. U0126 and BDNF were applied as indicated in Fig. 7A. To quantify Western blot, normalization using the response to exogenous BDNF application was performed. Data represent the mean ± SD (n = 5).

[a] $P < 0.01$ (none *vs.* BDNF), Kruskal-Wallis test and Mann-Whitney *U* test.

[b] $P < 0.01$ (BDNF *vs.* BDNF plus U0126), Kruskal-Wallis test and Mann-Whitney *U* test.

Table 2. Effect of U0126 on the BDNF-Increased Synaptic Proteins

	None	BDNF	BDNF + U0126	U0126
NR2A	0.28 ± 0.09	1.00[a]	0.52 ± 0.22[b]	0.29 ± 0.13
NR2B	0.38 ± 0.14	1.00[a]	0.65 ± 0.15[b]	0.45 ± 0.12
GluR1	0.41 ± 0.29	1.00[a]	0.68 ± 0.16[b]	0.23 ± 0.21
Synapsin I	0.67 ± 0.25	1.00[a]	0.65 ± 0.16[b]	0.54 ± 0.19
TUJ1	0.94 ± 0.16	1.00	1.12 ± 0.13	1.06 ± 0.14

U0126 and BDNF were applied as indicated in Fig. 7A. To quantify Western blotting, normalization using the response to exogenous BDNF application was performed. BDNF-induced increases in NR2A, NR2B, GluR1, and synapsin I were prevented by U0126. As a control, TUJ1 is also shown. Data represent the mean ± SD (n = 5).

[a] $P < 0.01$ (none *vs.* BDNF), Kruskal-Wallis test and Mann-Whitney *U* test.

[b] $P < 0.01$ (BDNF *vs.* BDNF plus U0126), Kruskal-Wallis test and Mann-Whitney *U* test.

DISCUSSION

In the present study, we found that DEX disturbed BDNF-mediated synaptic maturation in developing hippocampal neurons. Pretreatment with DEX inhibited BDNF-reinforced neurite outgrowth and synaptic protein expression in the early development stage. In the more mature stage, the inhibitory effect of DEX on synaptic function increased by BDNF was also observed. Interestingly, the reduction in the BDNF-activated MAPK/ERK pathway, a required intracellular signaling for BDNF-increased synaptic proteins, was observed after DEX exposure. Thus, it is possible that BDNF-mediated synaptic maturation is suppressed via reducing activation of the MAPK pathway when young neurons are exposed to high-level glucocorticoid during their development stage.

Morphological change of mature neurons seems to depend on the duration of stress hormone exposure. Chronic stress and administration of glucocorticoid resulted in dendritic atrophy of hippocampal neurons (10–13). Dendrite atrophy of the medial prefrontal cortex of adult rat was also observed after mild and short stress (14). Immature neurons might be more sensitive to stressful stimulation than mature neurons. Indeed, maternal separated infants during P2–P14 exhibited increase of anxiety, learning deficits, and decreased mossy fiber in the CA3 region of the hippocampus after they grew up (15). In our system, the number of neurites was estimated 3 d after starting DEX exposure with or without BDNF addition. Sole DEX exposure did not alter the number of neurites, although the inhibitory effect on the BDNF-increased neurite outgrowth was significant. The 3-d exposure to DEX may be a relatively shorter duration of exposure. If young neurons are exposed for a longer period, significant dendrite atrophy may be induced by sole DEX exposure.

BDNF is a key molecule involved in many neuronal aspects of developing and mature neurons. In particular, BDNF promotes the outgrowth of neurites and increases the expression of synaptic proteins, which are required for establishing synaptic connections or functions during the development stage (24). In hippocampal slice cultures prepared from P7 rats, treatment with BDNF for 48 h increased synaptic vesicle proteins, such as synaptotagmin, synaptophysin, and synaptobrevin (29). Exercise-induced increase in the expression of synapsin I and synaptophysin in the hippocampus has been reported, and this increase in synaptic protein expression depended on BDNF (37). In dissociated cortical neurons, we recently reported that chronic treatment with BDNF enhanced the expression of synapsin I, synaptotagmin, and synaptophysin (26). These results suggest that BDNF is an essential molecule for establishing the basal machinery of synaptic function and plasticity in CNS neurons. Consistently, in the present study, BDNF significantly increased the expression of presynaptic proteins including synapsin I, synaptotagmin, and SNAP25 in