of cortical interneurons from the ventral to the dorsal telencephalon in rodents (Powell et al., 2001), and HGF-MET signaling systems are implicated in regulating the proliferation and differentiation of cerebellar granule cells (Ieraci et al., 2002). Furthermore, HGF plays a role in regulating the morphology of cortical pyramidal dendrites in the early postnatal period, and endogenous levels of HGF are necessary for the normal development of these neurons (Gutierrez et al., 2004). Taken together, these findings suggest that HGF may be a candidate for mediating interneuron development *in vivo* (Levitt et al., 2004).

Given the key role of HGF in brain development, it is of great interest to study the role of HGF in the pathophysiology of autism. In this study, we therefore studied whether serum HGF levels in subjects with high-functioning autism are altered as compared with age-matched healthy controls. Furthermore, we also examined the relationship between serum HGF levels and clinical symptoms in subjects with autism.

2. Methods

2.1. Subjects

This study was approved by the Ethics Committee of the Hamamatsu University School of Medicine. After the participants were given a complete description of the study, written informed consent was obtained from all subjects before they entered the study.

One of the authors (M.T.) coordinates a self-help group for subjects with autism and their families, "Asupe-erude-no-kai" (the Association for Asperger Syndrome and Learning Disorders), in Nagoya, Japan. Seventeen male subjects with high-functioning autism were recruited from this group and enrolled in this study. The diagnosis of autism was made on the basis of the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1994), Japanese version. One of the authors (K.J.T.) having established reliability of diagnosing autism with the authors conducted the interview for all subjects, and then, based on the results, a DSM-IV (American Psychiatric Association, 1994) diagnosis of Autistic Disorder was made for all subjects. ADI-R is a semi-specially-formulated structured psychiatric interview with a parent, especially a mother, which is administered to the parent. It is used to confirm diagnosis and also to evaluate the core symptoms of autism. ADI-R is based on three separate scores. ADI-R domain score A quantifies impairment in social interaction (the range of score: 0–32), domain score BV quantifies impairment in communication (the range of score: 0–26), and domain score C quantifies restricted, repetitive, and stereotyped patterns of behavior and interests (the range of score: 0–16). Higher scores on each indicate worse performance. The cut-off scores of domain score A, domain score BV, and domain score C are 10, 8, and 3, respectively. All of the subjects with autism have scores above the cut-off scores. ADI-R domain D corresponds to age of onset criterion for autistic disorder. If the score is 1 or higher, the subject is quite likely to have the age of onset prior to 3 year old. All of the subjects with autism have age of onset no later than 3 year old since none has ADI-R domain D score lower than 1 (Table 1).

Table 1
Clinical characteristics of normal controls and subjects with high-functioning autism

Group (n)	Control (18)	High-functioning autism (17)
Age (year)	23.0±2.03 (20–26)	23.1±2.52 (19–28)
HGF (pg/mL)	817.6±232.4 (552–1243)	503.5±160.5 (302–841) ***
ADI-R		
Domain A score, social		22.5±4.8 (14–29)
Domain BV score, communication		16.5±3.8 (9–22)
Domain C score, stereotype		5.3±1.8 (3–10)
Domain D score, age of onset		3.7±1.1 (1–5)
Y-BOCS (total score)		11.2±5.6 (2–26)
Hamilton Depression Scale score		2.4±3.7 (0–15)
Hamilton Anxiety Scale score		4.1±3.3 (0–11)
AQ total score		50.6±12.7 (34–69)
Faux Pas test — Theory of Mind		23.4±8.8 (3–34)
WAIS-R (full-scale IQ)		98.9±18.9 (71–140)
Gestational age (week)[a]		38.8±1.7 (34–41)
Birth weight (g)[a]		3382±502 (2376–4148)
Head circumference at birth (cm)[a]		34.0±2.3 (29.2–37.6)

ADI-R: Autism Diagnostic Interview-Revised, Y-BOCS: Yale–Brown Obsessive Compulsive Scale, AQ: Aggression Questionnaire, WAIS-R: Wechsler Adult Intelligence Scale-Revised.
Values are expressed as mean±SD (range).
*** $p<0.001$ as compared to control (the Mann-Whitney U test).
[a] One subject had no available information.

We also conducted the WAIS-R to exclude subjects with a full-scale Intelligence Quotient (IQ) of less than 70, resulting in a group of 17 subjects with high-functioning autism. Participants were excluded from the study, if they had a diagnosis of fragile X syndrome, epileptic seizures, obsessive–compulsive disorder, affective disorders, or any additional psychiatric or neurological diagnoses. All the subjects with autism were drug naive or free of psychoactive medications for at least 6 months: the majority of participants with autism have never previously received psychoactive medications, and the minority participants had been given sedatives more than 6 months before this study. Eighteen subjects who had no developmental delay and no history of psychiatric disorders or treatment joined our study as controls. All control group participants underwent a comprehensive assessment of medical history to eliminate individuals with any neurological or other medical disorders. None of the comparison subjects initially recruited was found to fulfill these exclusion procedures. There was no significant age difference between the control subjects and the subjects diagnosed with high-functioning autism (Table 1). All participants for both groups were Japanese.

2.2. Psychological measures

In addition to the IQ and ADI-R assessments, we adopted the Yale–Brown Obsessive Compulsive Scale (Y-BOCS) (Goodman

et al., 1989a,b), the Aggression Questionnaire (Buss and Perry, 1992), the Faux Pas test (Baron-Cohen et al., 1999; Stone et al., 2003), the Hamilton Depression Scale (HAM-D) (Hamilton, 1960), and the Hamilton Anxiety Scale (HAM-A) (Hamilton, 1969) to evaluate clinical and psychological correlates of the subjects with high-functioning autism. To extract early developmental factors, we made use of the Mother and Child Health Handbook (MCHH). The MCHH is a special notebook provided to each pregnant woman in Japan for recording the child's medical information throughout pregnancy, delivery, and early development. Subjects with high-functioning autism were asked to submit the MCHH, which was used to collect information on gestational week, birth weight and head circumference at birth.

2.3. Measurement of serum HGF levels

Serum samples of subjects with autism and normal comparison subjects were collected during 11:00-noon, and stored at −80 °C until assay. Serum HGF levels were measured using HGF enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, Inc., Minneapolis, MN). The assay was performed according to the supplier's recommendation. The calibrator consisted of recombinant human HGF. All samples were measured in duplicates and respective mean value was calculated.

2.4. Statistical analysis

The data were presented as the mean±standard deviation (SD). The data were analyzed using the Mann–Whitney U test. The relationship between HGF levels and clinical variables among subjects with autism was determined by Spearman correlations. A p value of less than 0.05 was considered to be statistically significant.

3. Results

The serum levels (503.5±160.5 pg/mL) of HGF in the subjects with high-functioning autism were significantly (Mann–Whitney $U=34.0, p<0.001$) lower than those (817.6±232.4 pg/mL) of normal controls (Table 1 and Fig. 1). However, there were no marked or significant correlations between serum HGF levels and clinical variables, including ADI-R subscale scores, Y-BOCS total score, Hamilton Depression Scale score, Hamilton Anxiety Scale score, Aggression Questionnaire total score, Faux Pas test score, Full-scale IQ, gestational age, birth weight, and head circumference at birth (Table 1).

4. Discussion

In this study, we found that serum HGF levels in subjects with high-functioning autism were significantly lower than those of age-matched normal controls. To the best of our knowledge, this is the first report demonstrating the decreased serum levels of HGF in subjects with autism. Although it remains unclear whether serum HGF levels reflect the levels of HGF in the brain, it is possible that decreased levels of HGF may occur in the brain of subjects with high-functioning autism since exogenous HGF prevented neuronal cell death in the hippocampal CA1 region after transient global ischemia (Miyazawa et al., 1998). Recently, it has been reported that cerebrospinal fluid levels of HGF in subjects with autism (aged 3–10 years, $n=6$) are significantly higher than those of normal control (aged 12–45 years, $n=9$) (Vargas et al., 2005). However, age of subjects enrolled in that paper was not matched to normal controls (Vargas et al., 2005), indicating that further studies using age-matched large samples are necessary. Given the key role of HGF in brain development (Maina and Klein, 1999; Levitt et al., 2004), our findings lead us to the hypothesis that decreased levels of HGF in the brain may contribute to the pathophysiology of autism. In this study, serum HGF levels between autistic subjects and controls were overlapped. Furthermore, we did not find any correlations between serum HGF levels and clinical variables in the autistic subjects, suggesting that serum HGF levels may be a trait marker, not a state marker, in subjects with autism. Nonetheless, it may be of interest to measure serum HGF levels in children with and without autism in order to determine the role of HGF as a serological marker in children who will go on to develop an autistic disorder. In addition, the mechanism by which decreased HGF levels play a role in the pathophysiology of autism remains to be established.

Subjects with autism are at greater risk for developing seizure disorders, particularly in adolescence (Tuchman and Rapin, 2002; Volkmar and Pauls, 2003). Multiple lines of evidence suggest that seizure is caused by imbalance excitatory and inhibitory

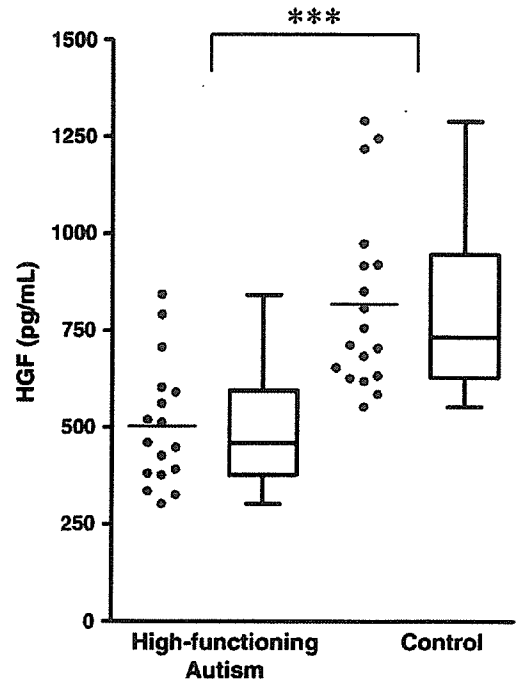

Fig. 1. The serum levels of HGF in normal controls and subjects with high-functioning autism. The serum levels of HGF in subjects with autism ($n=17$) were significantly lower than those of normal controls ($n=18$). The bar in the dot plot shows the mean values. The boxes represent the median, the 25th and 75th percentiles. Lines outside the boxes represent the 10th and 90th percentiles (minimum and maximum limits, respectively). ***$p<0.001$ (Mann–Whitney U test).

neurotransmitter systems in brain, and that γ-aminobutyric acid (GABA) interneurons play a critical role in maintaining this balance (Levitt et al., 2004). The pathophysiology in autism is currently unknown, but there is interesting convergence of data to suggest a regional disruption of interneuron development (Levitt et al., 2004). Mice with a targeted mutation of the gene encoding urokinase plasminogen activator receptor (uPAR), a key component in HGF activation and function, have decreased levels of HGF and a 50% reduction in neocortical GABAergic interneurons at embryonic and perinatal ages (Powell et al., 2003). Behavioral test of *uPAR* (−/−) mice showed increased anxiety in three paradigms (open field, light–dark exploration, and the elevated plus maze), and spontaneous myoclonic seizures and a greater susceptibility to pharmacologically induced convulsion (Powell et al., 2003). Furthermore, the mutation of *uPAR* gene results in interneuron loss and behaviors similar to human epilepsy, autism and anxiety disorders (Powell et al., 2003; Levitt et al., 2004). These findings suggest that disruption of the HGF-MET signaling systems results in complex alterations in GABAergic interneuron development in the forebrain (Levitt et al., 2004). Taken together, it is likely that decreased HGF levels may be implicated in the high rates of seizure disorder in autism although further studies on the role of HGF-MET signaling systems on the high rate of seizure in autism are required for investigation of its pathological roles in autism.

5. Conclusion

In conclusion, this study suggests that reduced HGF levels might be implicated in the pathophysiology of high-functioning autism. Further studies on the molecular and cellular implications of HGF in the pathophysiology of autism will be necessary.

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Genetic analyses of the brain-derived neurotrophic factor (BDNF) gene in autism

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Abstract

Autism is a severe neurodevelopmental disorder defined by social and communication deficits and ritualistic-repetitive behaviors that are detectable in early childhood. Brain-derived neurotrophic factor (BDNF) plays a critical role in the pathogenesis of autism. In this study, we examined the SNP- and haplotypic-association of BDNF with autism in a trios-based association study (the Autism Genetic Resource Exchange). We also examined the expression of BDNF mRNA in the peripheral blood lymphocytes of drug-naïve autism patients and control subjects. In the TDT of autism trios, the SNP haplotype combinations showed significant associations in the autism group. BDNF expression in the drug-naïve autistic group was found to be significantly higher than in the control group. We suggest that BDNF has a possible role in the pathogenesis of autism through its neurotrophic effects on the serotonergic system.
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Keywords: Autism; Brain-derived neurotrophic factor; Serotonin transporter; A trios-based association; Peripheral blood lymphocytes

Autism is a severe neurodevelopmental disorder defined by social and communication deficits and ritualistic-repetitive behaviors that are detectable in early childhood. The serotonergic system has been found to be developmentally dysregulated in autism; factors that regulate serotonergic neuronal development and serotonin metabolism might have a crucial role in the pathophysiology of autistic disorders caused by the dysfunction of the serotonergic system [1]. Specifically, altered developmental dynamics of serotonin synthesis [2,3] and increases in whole blood serotonin levels have been reported in autistic individuals [4,5]. Effective medications for treating autistic symptoms include drugs that have an impact on the serotonergic system, such as the serotonin receptor antagonists (e.g. Risperidone) and serotonin depleting agents (e.g. Fenfluramine) [6–8].

Multiple lines of evidence suggest that brain-derived neurotrophic factor (BDNF) plays a critical role in the serotonergic function. In the rat brain, BDNF has been found to promote the survival and sprouting of serotonergic axons [9] and the axonal growth of injured serotonergic neurons

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[10,11]. In vitro and in vivo studies support a regulatory role of BDNF in the survival and maturation of serotonergic neurons [12,13]; BDNF has also been shown to modulate serotonergic neurotransmission in vitro [14]. In addition, BDNF administration has been found to increase the synthesis and/or turnover of serotonin in vivo [15–18].

BDNF has a detrimental effect on the aforementioned processes, and has been implicated in the pathogenesis of neurodevelopmental disorders like autism. Specifically, elevated BDNF expression has been observed in the brain [19], blood [20] and serum [21,22] of autistic individuals, compared to healthy controls. Recently, we found that the serum levels of BDNF in patients with autism were significantly lower than those of normal controls [23].

In this study, we examined the SNP- and haplotypic-association of BDNF with autism in a trios-based association study. We also examined the expression of BDNF mRNA in the peripheral blood lymphocytes of drug-naïve autism patients and control subjects, since lymphocytes are now considered to be a convenient and accessible alternative to brain samples for use in biochemical and genetic investigations of the functions of the central nervous system [24].

Materials and methods

Association study

Subjects. The study was approved by the Ethics Committee of the Hamamatsu University School of Medicine.

DNA samples from trios families recruited to the Autism Genetic Resource Exchange (AGRE; http://www.agre.org) were used for this study [25]. We selected trios families, with male offspring scored for autism; additional selection criteria required that (i) there be no possible non-idiopathic autism flag and (ii) all the trios be Whites. Two sets of samples were used in this study; the first set consisted of 104 high-functioning autism (HFA) trios, with the affected offspring having an intelligence quotient (IQ) > 70, whereas, the second set consisted of randomly chosen trios with no IQ information.

Genotyping. The genomic structure of BDNF is based on the UCSC May 2004 draft assembly of the human genome (http://www.genome.ucsc.edu). The BDNF gene is located in 11p14, spanning a genomic stretch of 66.8 kb (mRNA 1580 bases). SNPs were selected based on information from the National Centre for Biotechnology Information (NCBI dbSNP: http://www.ncbi.nlm.nih.gov/SNP), The SNP Consortium (TSC: http://www.snp.cshl.org) and the International HapMap Project (http://www.hapmap.org). On the basis of their genomic locations and the minor allele frequencies (MAF) in the Caucasian population, 25 SNPs were chosen for our analysis in order to span the BDNF gene as evenly as possible. Assay-on-demand/Assay-by-design SNP genotyping products (Applied Biosystems, Foster City, CA, USA) were used to score the SNPs based on the TaqMan assay method [26]. Genotypes were determined using the ABI 7900 Sequence Detection System (SDS) (Applied Biosystems) and analyzed using SDS v2.0 software (Applied Biosystems). The SNPs used in the study and their locations are shown in Table 1.

Statistical analysis. PedCheck program v1.1 (http://www.watson.hgen.pitt.edu) was used to identify and eliminate all Mendelian inconsistencies in the trios data set. Markers were tested for association by the conventional transmission disequilibrium test (TDT) using the TDTPHASE program of the UNPHASED software package v2.403 (http://www.hgmp.mrc.ac.uk). All of the three-, four-, and five-marker haplotypes were tested for association in a sliding window across the locus. The option 'drop rare haplotypes' was used to restrict the analysis of haplotypes with a frequency <3%. Pair-wise linkage disequilibrium (LD) between the various markers, based on D' (linkage disequilibrium coefficient) values [27], was estimated using the Haploview software v3.2 (http://www.broad.mit.edu/mpg/haploview); an LD plot was also constructed using this software.

Gene expression analysis

Lymphocyte RNA. The study was approved by the Ethics Committee of the Hamamatsu University School of Medicine. Written informed consent was obtained from each participant after having been provided an explanation of the study procedures. We obtained blood samples from 11 drug-naïve autism patients (age 21.4 ± 2.31 years [mean ± SD]) and 13 age-matched (22.3 ± 1.93) healthy controls. All the patients and control subjects were males, and were of Japanese origin.

The autism patients were diagnosed according to the Autism Diagnostic Interview-Revised (ADI-R) by trained and certified psychiatrists (K.T.,A.S.) [28]. All of the patients met the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria (American Psychiatric Association, 1994) [29] and International Classification of Diseases, 10th Revision (ICD-10; World Health Organization, 1992) [30], criteria for autism. The patients underwent screening, and were excluded if they had any major medical- or psychiatric-conditions; they had been drug-naïve for at least 6 months. Comorbid anxiety and depressive symptoms were assessed using the Hamilton Anxiety Rating Scale (HAM-A) [31] and the Hamilton Depression rating scale (HAM-D) [32], respectively. Obsessional/repetitive behaviors were clinically rated using the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) [33,34]. Additional aggression symptoms were also assessed using the aggression questionnaire (AQ) [35]. A faux pas detection task was used to measure theory of mind (ToM) [36,37]. All of the evaluations were conducted by a trained research psychiatrist (K.N.).

All of the controls were free of medications, and underwent screening to exclude neurological-, developmental-, or psychiatric-disorders and mental retardation; none of them met any of the relevant criteria of DSM-IV.

Peripheral blood (20 ml) was drawn from the cubital vein into EDTA-containing plastic syringes. Lymphocytes were isolated from blood samples by the Ficoll-Paque gradient method, and total RNA was extracted using RNAzolB reagent (Sawady, Tokyo, Japan) according to the manufacturer's instructions. RNA samples were quantified by analyzing the absorbance at 260 nm in a UV-spectrophotometer. Complementary DNA (cDNA) was synthesized by first strand reverse transcriptase reaction (RT) using Random Primer and M-MLV reverse transcriptase (Invitrogen, CA, USA).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR): Real-time qRT-PCR analysis was performed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). TaqMan primer/probes for BDNF and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as the endogenous reference, were purchased from Applied Biosystems (Assay-on-Demand™ gene expression products Hs00156058 and Hs99999905, respectively). All reactions were performed in duplicate according to the manufacturer's protocol. A comparative threshold cycle (C_T) method validation experiment was done to check whether the efficiencies of the target and reference amplifications were approximately equal (the slope of the log input amount versus $\Delta C_T < 0.1$). One sample was randomly chosen as a calibrator, and was amplified in each plate to correct for experimental differences among consecutive PCR runs. The amounts of BDNF mRNA were normalized to the endogenous reference, and were expressed relative to the calibrator as $2^{-\Delta\Delta C_T}$ (comparative C_T method).

Statistical analysis. Statistical calculations were performed using SPSS statistical package, version 11.0.1 (SPSS Co. Ltd., Tokyo, Japan) and GraphPad Prism, version 4.00 (GraphPad Software, San Diego, CA, USA). The difference in BDNF expression between the groups was analyzed using the t-test. The correlation between various clinical features and BDNF expression was examined using Pearson's correlation coefficient.

Table 1
Single SNP TDT results of BDNF SNPs

Marker	dbSNP ID	Variation[a]	Location (NCBI B34)	Minor allele frequency[b]	HFA trios (IQ > 70)		Random trios	
					T (%)[c]	*p*-value	T (%)[c]	*p*-value
SNP01	rs1491851	A/G	27717072	0.459	47.77	0.4	50.72	0.753
SNP02	rs727155	G/A	27714758	0.044	50.25	0.586	50	1
SNP03	rs1491850	A/G	27714034	0.436	47.66	0.226	50.6	0.74
SNP04	rs908867	G/A	27710073	0.093	49.73	0.75	50.47	0.479
SNP05	rs12273363	A/G	27709168	0.166	50.14	0.893	50.4	0.658
SNP06	rs11030121	G/A	27700516	0.266	49.92	0.961	50.26	0.832
SNP07	rs7934165	C/T	27696292	0.473	52.91	0.239	49.68	0.869
SNP08	rs2030324	T/C	27691224	0.473	52.68	0.281	49.68	0.869
SNP09	rs988748	C/G	27689054	0.264	52.69	0.567	50.18	0.887
SNP10	rs2049046	A/T	27688084	0.458	53.3	0.17	49.29	0.708
SNP11	rs7127507	A/G	27679193	0.28	49.65	0.83	50.59	0.646
SNP12	rs7103411	A/G	27664434	0.264	49.21	0.545	50.03	0.98
SNP13	rs2049045	C/G	27658550	0.226	51.88	0.719	50.18	0.869
SNP14	rs1401635	C/G	27658300	0.268	52.73	0.495	50.61	0.625
SNP15	rs11030104	T/C	27648826	0.256	49.41	0.642	50.37	0.759
SNP16	rs6265 (V66M)	G/A	27644225	0.226	49.7	0.799	49.94	0.957
SNP17	rs7124442	A/G	27641350	0.27	48.97	0.51	50	1
SNP18	rs1519480	A/G	27640021	0.274	49.07	0.554	50.23	0.854
SNP19	rs4923463	T/C	27636809	0.259	49.38	0.624	50.37	0.76
SNP20	rs2203877	A/G	27635219	0.461	53.59	0.141	49.22	0.68
SNP21	rs10501087	A/G	27634417	0.259	49.38	0.624	50.37	0.76
SNP22	rs1519479	G/A	27631840	0.468	54.27	0.092	49.28	0.709
SNP23	rs925946	C/A	27631511	0.263	48.16	0.212	50.37	0.76
SNP24	rs11030096	T/C	27629852	0.461	54.19	0.092	49.29	0.708
SNP25	rs4923461	A/G	27621219	0.256	49.41	0.642	50.14	0.906

HFA, high-functioning autism; T, transmitted.
[a] Common allele is listed first.
[b] Based on the parental genotypes of random trios.
[c] T percentage of common allele is listed.

Results

Association study

Single SNP TDT

TDT was done separately for the HFA trios and for the random trios; the results are shown in Table 1. None of the SNPs showed a significant association in the HFA trios or random trios.

Haplotype TDT

The results of haplotype TDT for HFA- and random trios are shown in Table 2. The three-SNP haplotype combination of SNP04-SNP05-SNP06 ($p = 0.017$), the four-SNP haplotype combination of SNP04-SNP05-SNP06-SNP07 ($p = 0.02$) and the five-SNP haplotype combination of SNP04-SNP05-SNP06-SNP07-SNP08 ($p = 0.02$) showed significant associations in the random group; however, the global values were not significant. None of the three-, four- or five-SNP haplotypes showed significant association in the HFA trios.

LD analysis

LD analysis identified a single haplotype block across the BDNF gene, comprising SNPs 03–25 (Fig. 1).

Lymphocyte gene expression analysis: BDNF expression in the drug-naïve autistic (0.094 ± 0.1 [mean ± SD]) group was found to be significantly higher than in the control (0.034 ± 0.02) group ($t = -2.2$; $df = 22$; $p = 0.039$) (Fig. 2). No significant correlation was observed between any of the clinical features and BDNF expression in the autistic group (Table 3).

Discussion

In the present study, we reported the haplotypic association of BDNF with autism; three-, four-, and five-SNP haplotypes consisting of SNP04 (rs908867), SNP05 (rs12273363) and SNP06 (rs11030121) showed significant associations with random trios. Furthermore, we found that BDNF expression in the drug-naïve autism group was significantly higher than in the control group. To the best of our knowledge, this is the first report demonstrating an association and increased BDNF expression in drug-naïve autism subjects.

The BDNF Val66Met polymorphism (SNP16 in this study) has been reported to be associated with obsessive-compulsive disorder [38], attention deficit hyperactivity disorder [39] and anxiety-related personality traits [40]; this SNP has also been suggested to have a role in the

Table 2
Three-, four- and five-SNP haplotype analysis of BDNF

Three-SNP[a]	*p*-value[b]		Four-SNP[a]	*p*-value[b]		Five- SNP[a]	*p*-value[b]	
	HFA trios	Random trios		HFA trios	Random trios		HFA trios	Random trios
1-2-3	0.151	0.863	1-2-3-4	0.151	0.947	1-2-3-4-5	0.197	0.931
2-3-4	0.658	0.698	2-3-4-5	0.73	0.8	2-3-4-5-6	0.67	0.887
3-4-5	0.499	0.822	3-4-5-6	0.622	0.068	3-4-5-6-7	0.531	0.071
4-5-6	0.698	***0.017***	4-5-6-7	0.576	***0.02***	4-5-6-7-8	0.611	***0.02***
5-6-7	0.721	0.937	5-6-7-8	0.766	0.937	5-6-7-8-9	0.833	0.914
6-7-8	0.636	0.98	6-7-8-9	0.717	0.987	6-7-8-9-10	0.52	0.968
7-8-9	0.635	0.987	7-8-9-10	0.47	0.968	7-8-9-10-11	0.52	0.956
8-9-10	0.49	0.968	8-9-10-11	0.539	0.956	8-9-10-11-12	0.539	0.965
9-10-11	0.634	0.918	9-10-11-12	0.634	0.932	9-10-11-12-13	0.752	0.996
10-11-12	0.634	0.932	10-11-12-13	0.752	0.996	10-11-12-13-14	0.565	0.975
11-12-13	0.702	0.985	11-12-13-14	0.53	0.958	11-12-13-14-15	0.547	0.963
12-13-14	0.538	0.948	12-13-14-15	0.586	0.95	12-13-14-15-16	0.612	0.907
13-14-15	0.681	0.933	13-14-15-16	0.659	0.892	13-14-15-16-17	0.193	0.943
14-15-16	0.686	0.89	14-15-16-17	0.201	0.94	14-15-16-17-18	0.234	0.922
15-16-17	0.555	0.966	15-16-17-18	0.601	0.958	15-16-17-18-19	0.596	0.958
16-17-18	0.582	0.998	16-17-18-19	0.596	0.964	16-17-18-19-20	0.61	0.966
17-18-19	0.43	0.931	17-18-19-20	0.444	0.92	17-18-19-20-21	0.444	0.92
18-19-20	0.4	0.916	18-19-20-21	0.4	0.916	18-19-20-21-22	0.447	0.916
19-20-21	0.352	0.912	19-20-21-22	0.41	0.912	19-20-21-22-23	0.42	0.822
20-21-22	0.41	0.912	20-21-22-23	0.42	0.822	20-21-22-23-24	0.42	0.822
21-22-23	0.32	0.829	21-22-23-24	0.314	0.829	21-22-23-24-25	0.314	0.845
22-23-24	0.253	0.926	22-23-24-25	0.314	0.985			
23-24-25	0.305	0.985						

[a] Based on all possible haplotypes for each combination of SNPs.
[b] Computed on the basis of likelihood ratio test. Significant *p*-values (<0.05) are indicated in bold italics.

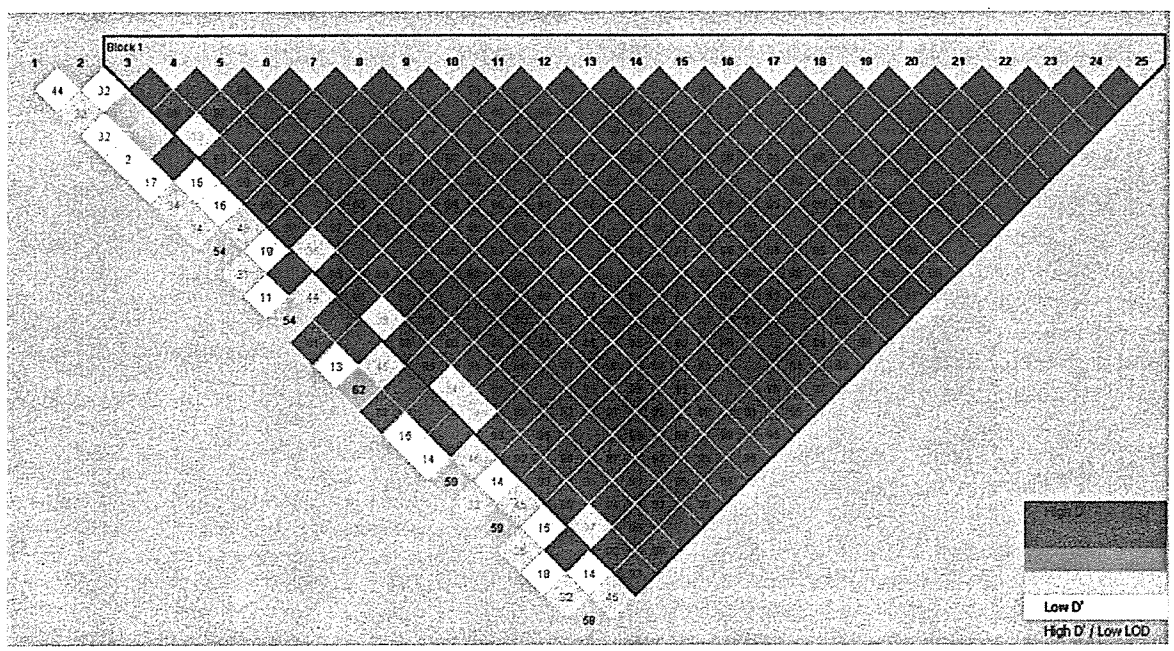

Fig. 1. Haplotype block structure of BDNF based on D' values calculated from 148 random trios.

hippocampal and prefrontal cortex functions involved in human memory and learning [41,42]. However, we did not observe any significant association of Val66Met with autism.

Several lines of evidence suggest that BDNF hyperactivity can be deleterious to the neurodevelopmental process, and it has been implicated in the pathophysiology of neurodevelopmental disorders like autism. Specifically, Nelson et al. [20] reported higher BDNF levels in the archived samples of neonatal blood from autistic children compared to normal controls. This finding was further supported by observations of higher concentrations of BDNF in the

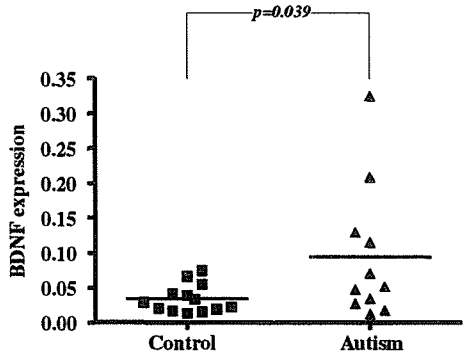

Fig. 2. *t*-test comparison of BDNF mRNA levels in the lymphocytes from control subjects and drug-naïve autism patients. Horizontal bars indicate means. A significant difference in BDNF expression was observed between the two groups ($p = 0.039$).

Table 3
Correlation between clinical features and lymphocyte BDNF expression in the autism group

Clinical feature	Pearson (*r*)	*p*-value
HAM-A	−0.199	0.581
HAM-D	−0.223	0.536
Y-BOCS	−0.141	0.699
Obsession	−0.223	0.536
Compulsion	−0.046	0.899
Aggression questionnaire	0.185	0.610
Faux pas test	0.231	0.530

basal forebrain [19] and in the serum [21,22] of autistic patients compared to healthy controls. In this present study, enhanced BDNF m-RNA expression in the lymphocytes was observed in the drug-naïve autistic group compared to the control group. Therefore, BDNF hyperactivity could result in disruption of the normal developmental program in the brain, leading to abnormalities like overgrowth of brain- and neuronal-tissues, which has been observed in autistic individuals [43–47].

There was no significant correlation between BDNF expression and any of the clinical features of the autistic group. Hence, it may be suggested that elevated BDNF expression is indicative of the disease state per se, and is not dependent on the clinical features of the disease.

Since BDNF has a proven role in regulating the structural [9–13] and functional aspects [14–18] of serotonergic neurons, its hyperactivity might cause dysfunction of the serotonergic system. In a B lymphoblast model, which had several molecular and functional similarities to serotonergic neurons, BDNF treatment was found to decrease serotonin uptake by serotonin transporters, thereby increasing extracellular serotonin levels [48].

Given the critical role of BDNF in brain development, our findings lead us to the hypothesis that enhanced levels of BDNF may contribute to the pathophysiology of autism. It is therefore of great interest to measure BDNF levels in children with autism in order to determine the role of BDNF as a serological marker in children who will go on to develop an autistic disorder.

In conclusion, we suggest that BDNF hyperactivity may play a role in the pathogenesis of autism through its neurotrophic effects on the serotonergic system. Moreover, this is the first report of a genetic association between BDNF and autism; however, replication of these findings and further studies of the functional impact of BDNF in autism are warranted.

Acknowledgments

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Decreased Serum Levels of Platelet-Endothelial Adhesion Molecule (PECAM-1) in Subjects with High-Functioning Autism: A Negative Correlation with Head Circumference at Birth

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Background: Accumulating evidence suggests that the immune system plays a role in the pathophysiology of autism, and that the adhesion molecules play an important role in the process of inflammation. This study was undertaken to determine whether serum levels of the adhesion molecules in subjects with high-functioning autism are altered as compared with those of normal controls.

Methods: Seventeen male subjects with high-functioning autism and 22 male age-matched unrelated healthy control subjects were enrolled. Serum levels of the soluble forms of platelet-endothelial adhesion molecule (PECAM-1), intracellular adhesion molecule (ICAM-1), and vascular cell adhesion molecule (VCAM-1) were measured.

Results: Levels of PECAM-1, but not ICAM-1, in the subjects with autism were significantly lower than those of control subjects. VCAM-1 showed a weak trend for a lowered level. There was a negative correlation between serum levels of PECAM-1 and head circumference at birth in the autistic subjects.

Conclusions: These results suggest that PECAM-1 plays a role in the pathophysiology of high-functioning autism.

Key Words: Adhesion molecules, developmental disorders, ELISA, head circumference, high-functioning autism, human blood

Autism is a developmental disorder that is characterized by severe impairment in social interaction and communication and by the presence of stereotyped behaviors. The mechanisms underlying the pathophysiology of this disorder remain to be determined (Baron-Cohen and Belmonte 2005; Volkmar and Pauls 2003). However, accumulating evidence suggests that the immune system plays a role in the pathophysiology of autism (Cohly and Panja 2005; Krause *et al.* 2002; Pardo *et al.* 2005).

Adhesion molecules are localized both on the membranes of activated platelets and leukocytes and on the vascular endothelium. They mediate the binding of leukocytes to the blood vessel wall, which is the main step in the process of inflammation (Blankenberg *et al.* 2003; Lee and Benveniste 1999). Intracellular adhesion molecule ICAM-1 is widely expressed at a basal level and can be up-regulated by pro-inflammatory cytokines in leukocytes and endothelial cells. The vascular cell adhesion molecule VCAM-1 is transcriptionally induced on endothelial cells but can also be expressed by other cell types, such as macrophages, myoblasts, and dendritic cells. The platelet-endothelial adhesion molecule PECAM-1 is particularly dense at the junctions between endothelial cells, where it mainly participates in homophilic binding between adjacent cells. Circulating levels of soluble-forms of adhesion molecules have been shown to be increased in various inflammation-related diseases, such as atherosclerosis and multiple sclerosis (Blankenberg *et al.* 2003), although it is not known whether adhesion molecules play roles in the pathophysiology of other disorders occurring in central nervous system (CNS).

Considering the key role played by adhesion molecules in immune responses in the CNS (Blankenberg *et al.* 2003; Lee and Benveniste 1999), it would be of interest to study the role of adhesion molecules in the pathophysiology of autism. The purpose of this study was to examine whether serum levels of ICAM-1, VCAM-1, and PECAM-1 in subjects with high-functioning autism would be altered as compared to those in age-matched normal controls. In addition, we examined the relationships between serum levels of adhesion molecules and clinical variables in autistic subjects.

Methods and Materials

One of the authors (MT) coordinates a self-help group for subjects with autism and their families, "Asupe-erude-no-kai" (the Association for Asperger Syndrome and Learning Disorders), in Nagoya, Japan. Seventeen male subjects with high-functioning autism were recruited from this group and enrolled in this study. The diagnosis of autism was made on the basis of the Autism Diagnostic Interview-Revised (ADI-R)(Lord *et al.* 1994), Japanese version. One of the authors (KJT) having established reliability of diagnosing autism, conducted the interview for all subjects, and then, based on the results, a DSM-IV (American Psychiatric Association 1994) diagnosis of autistic disorder was made for all subjects. Five of the authors (KJT, YI, YS, GS, MK) confirmed,

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using a structured interview, that subjects with autism had no diagnosis other than autism and that control subjects had no psychiatric diagnosis. We also conducted the WAIS-R to exclude subjects with a full-scale IQ of less than 70, resulting in a group of 17 subjects with high-functioning autism. No other psychiatric disorder was found. Twenty-two subjects who had no developmental delay and no history of psychiatric disorders or treatment joined our study as controls. There was no significant age difference between the control subjects and the subjects diagnosed with high-functioning autism (Table 1). No subjects had such diagnoses although adhesion molecules were reported to be elevated in subjects with atherosclerosis and myocardial infarction. This study was approved by the Ethics Committee of the Hamamatsu University School of Medicine (Hamamatsu, Shizuoka). All participants were given a complete description of the study, and provided written informed consent before entry into the study.

In addition to the IQ and ADI-R assessments, we adopted the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS), the Aggression Questionnaire, the Faux Pas test, the Hamilton Depression Scale, and the Hamilton Anxiety Scale to evaluate clinical and psychological correlates of the subjects with high-functioning autism. To extract early developmental factors, we made use of the Mother and Child Health Handbook (MCHH), a special notebook provided to each pregnant woman in Japan for recording the child's medical information throughout pregnancy, delivery, and early development. Because the MCHH was recorded and written by an attending obstetrician and nurse contemporaneously, the information is free from recall biases. Subjects with high-functioning autism were asked to submit the MCHH, which was used to collect information on gestational week, birth weight at birth and head circumference at birth, and a summary score of pregnancy and obstetric complications as defined by Lewis *et al.* (1989).

Serum samples were obtained from the peripheral venous blood of the subjects between 11:00 am to noon after patients had fasted for at least 3 hours, and the samples were kept at room temperature for 30 min. They were centrifuged and divided into portions before stored at −80°C until use. Levels of ICAM-1, VCAM-1, and PECAM-1 were determined using the appropriate commercially available sandwich enzyme-linked immunosorbent assay kits (R & D Systems Inc., Minneapolis, Minnesota) according to the manufacturer's instructions. The sera of all subjects with high-functioning autism were measured together in one assay with a set of control sera. Each serum sample was analyzed in duplicate, and the mean value of the two measures was used for the analyses.

The data are presented as the mean ± standard deviation (SD). The data were analyzed using an unpaired *t*-test after confirming that there were no statistically significant differences in variance by an *F*-test. Evaluation of relationships between serum levels of adhesion molecules and clinical variables among subjects with high-functioning autism was performed with Spearman's rank-order correlation using the coefficient rho. A *p*-value of less than .01 was considered to be statistically significant for a conservative reason to avoid overestimation due to potential multiple correlations.

Results

VCAM-1 in autistic subjects showed a trend for a lowered level ($t = 2.53$, $df = 37$, $p = .016$) compared to control subjects (Table 1). Furthermore, the serum levels of PECAM-1 in autistic subjects were significantly ($t = 3.02$, $df = 37$, $p = .005$) lower than those in control subjects (Table 1). However, the serum levels of ICAM-1 in autistic subjects were not different ($t = 1.10$, $df = 37$, $p = .27$) from those of control subjects (Table 1).

We then examined the correlations between serum levels of these adhesion molecules and clinical variables among subjects with high-functioning autism. ADI-R subscale scores, which mainly suggest early diagnostic signs for autistic-spectrum disorders, did not show any correlations with levels of VCAM-1 or PECAM-1. Interestingly, we found a negative correlation (rho = −.683, $p = .007$) between serum PECAM-1 levels and head

Table 1. Clinical Characteristics of Normal Controls and Subjects with High-Functioning Autism

Group (*n*)	Control (22)	High-Functioning Autism (17)
Age (year)	23.1 ± 2.36 (18–26)	23.1 ± 2.52 (19–28)
ICAM-1 (ng/mL)	243.1 ± 52.7 (141.3–383.7)	224.4 ± 51.4 (104.0–278.8)
VCAM-1 (ng/mL)	640.6 ± 112.5 (476.9–929.0)	556.5 ± 88.3 (399.5–746.6)
PECAM-1 (ng/mL)	85.6 ± 18.5 (54.8–141.1)	69.6 ± 12.9 (47.4–98.8)[b]
ADI-R		
Domain A score, social		22.5 ± 4.8 (14–29)
Domain BV score, communication		16.5 ± 3.8 (9–22)
Domain C score, stereotype		5.3 ± 1.8 (3–10)
Domain D score, age of onset		3.7 ± 1.1 (1–5)
Y-BOCS (Total Score)		11.2 ± 5.6 (2–26)
Hamilton Depression Scale Score		2.4 ± 3.7 (0–15)
Hamilton Anxiety Scale Score		4.1 ± 3.3 (0–11)
AQ Total Score		50.6 ± 12.7 (34–69)
Faux Pas Test - Theory of Mind		23.4 ± 8.8 (3–34)
WAIS-R (Full-scale IQ)		989 ± 18.9 (71–140)
Lewis-Murray Scale Score[a]		.94 ± .77 (0–2)
Gestational Age (week)[a]		38.8 ± 1.7 (34–41)
Birth Weight (g)[a]		3382 ± 502 (2376–4148)
Head Circumference at Birth (cm)[a]		34.0 ± 2.3 (29.2–37.6)

Values are expressed as mean ± SD (range). ADI-R, Autism Diagnostic Interview-Revised; Y-BOCS, Yale-Brown Obsessive Compulsive Scale; AQ, Aggression Questionnaire; WAIS-R, Wechsler Adult Intelligence Scale-Revised.

[a]One subject had no available information.

[b]$p = .005$ as compared to control (unpaired *t*-test).

circumference at birth corrected for gestational age. Head circumference also showed a weak trend for negative correlation (rho = −.531, p = .051) with VCAM-1 levels. However, there were no marked or significant correlations between serum levels of VCAM-1 or PECAM-1 and other clinical variables, including Y-BOCS total score, Hamilton Depression Scale score, Hamilton Anxiety Scale score, Aggression Questionnaire total score, Faux Pas test score, full-scale IQ, Lewis-Murray Scale score, and gestational age and birth weight at birth.

Discussion

The major findings of the present study are that serum levels of PECAM-1 and VCAM-1 in subjects with high-functioning autism were lower than those of age-matched normal controls, and that serum levels of PECAM-1 were negatively correlated with head circumference at birth in autism. To the best of our knowledge, this is the first report demonstrating decreased serum levels of PECAM-1 and VCAM-1 in subjects with autism.

It remains unclear whether serum PECAM-1 levels reflect the levels of PECAM-1 in the brain. However, Zaremba and Losy (2002) reported that serum levels of PECAM-1 were significantly higher than cerebrospinal fluid (CSF) levels of PECAM-1 in acute ischemic stroke patients, and that serum levels of PECAM-1 were significantly correlated (r = .99, p < .0001) with CSF levels of PECAM-1, suggesting that the PECAM-1 fraction in CSF may be derived from blood (Zaremba and Losy 2002). Therefore, it is likely that decreased levels of PECAM-1 may occur in the brain of autistic patients. Given the key role of PECAM-1 in the process of inflammation (Blankenberg *et al.* 2003; Lee and Benveniste 1999), our findings led us to the hypothesis that decreased levels of PECAM-1 in the brain may contribute to the pathophysiology of autism. It is, therefore, of interest to measure serum level of PECAM-1 in very young children with and without autism in order to determine the role of these adhesion molecules as a serological marker in children who have just developed an autistic disorder. In addition, the mechanism by which decreased levels of these adhesion molecules play a role in the pathophysiology of autism remains to be established.

Several lines of evidence suggest that maternal characteristics and complications during pregnancy and at birth are associated with the development of autism (Glasson *et al.* 2004; Larsson et al 2005). Several studies have demonstrated the presence of brain enlargement during childhood in autism (Redcay and Courchesne 2005). A recent meta-analysis indicated that brain sizes in autism are substantially larger than normal during early childhood, with a peak enlargement of about 10% around 1 to 3 years of age (Redcay and Courchesne 2005). In this study, we do not know whether the head circumferences in the subjects with autism enrolled in this study were different from those of normal controls, since we did not collect the data of normal controls. However, we found a negative correlation between serum PECAM-1 levels and head circumference at birth in subjects with autism. It seems that decreased PECAM-1 levels may be implicated in the macrocephaly of autism, although it is currently unclear how decreased PECAM-1 levels affect brain sizes during childhood in autism. Further studies using a large sample will be necessary to unravel the relationship between PECAM-1 levels and macrocephaly in subjects with autism. Since VCAM-1 level showed trend for an inverse correlation with head circumference, this association deserves further attention to be explored as well as the association found in PECAM-1 level.

In conclusion, the present study suggests that reduced levels of adhesion molecules such as PECAM-1 might be implicated in the pathophysiology of autism. Further investigation into the physiological functions of these adhesion molecules in brain development may provide new insights regarding the role of the immune system in the pathophysiology of autism.

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Autistic-like phenotypes in *Cadps2*-knockout mice and aberrant *CADPS2* splicing in autistic patients

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Autism, characterized by profound impairment in social interactions and communicative skills, is the most common neurodevelopmental disorder, and its underlying molecular mechanisms remain unknown. Ca^{2+}-dependent activator protein for secretion 2 (CADPS2; also known as CAPS2) mediates the exocytosis of dense-core vesicles, and the human *CADPS2* is located within the autism susceptibility locus 1 on chromosome 7q. Here we show that *Cadps2*-knockout mice not only have impaired brain-derived neurotrophic factor release but also show autistic-like cellular and behavioral phenotypes. Moreover, we found an aberrant alternatively spliced *CADPS2* mRNA that lacks exon 3 in some autistic patients. Exon 3 was shown to encode the dynactin 1–binding domain and affect axonal CADPS2 protein distribution. Our results suggest that a disturbance in CADPS2-mediated neurotrophin release contributes to autism susceptibility.

Introduction

Autism is a severe neurodevelopmental disorder marked by profound disturbances in social, communicative, and behavioral functioning (1, 2). Epidemiological studies have shown that the prevalence of autism spectrum disorders is 3–6 per 1,000, with a male-to-female ratio of 3:1 (3). The concordance rate is about 90% in monozygotic twins and 10% in dizygotic twins (4), suggesting that autism has a prominent genetic component. Several genes, such as *neuroligin 3* (*NLGN3*), *NLGN4*, and *PTEN*, have been suggested to be associated with the development of autism (3, 5, 6). One susceptibility locus for autism was mapped to human chromosome 7q31-q33 (autism susceptibility locus 1 [AUTS1]) (7); however, none of the several candidate genes mapped to the AUTS1 locus have been directly implicated in autism (3).

Ca^{2+}-dependent activator protein for secretion 2 (CADPS2) is a member of the CAPS/CADPS protein family that regulates the exocytosis of dense-core vesicles at the ATP-dependent priming step by binding both phosphatidyl inositol 4,5-bisphosphate and dense-core vesicles (8). The human *CADPS2* is located on chromosome 7q31.32, within the AUTS1 locus (9). Mouse CADPS2 protein is associated with brain-derived neurotrophic factor–containing (BDNF-containing) secretory vesicles and is involved in the activity-dependent release of BDNF (10), and the cellular distribution of CADPS2 in the mouse brain largely coincides with that of BDNF (11). BDNF plays a key role in many aspects of brain development and function, including the formation of synapses and circuits (12, 13). However, the detailed mechanism of BDNF secretion remains elusive (14). A recent study indicates that the decreased level of BDNF expression in methyl CpG-binding protein 2–mutant (*Mecp2*-mutant) mice, a model of Rett syndrome (15), affects disease progression (16).

In this report, we describe the association of a deficit in CADPS2-mediated neurotrophin release with autism susceptibility by analyzing *Cadps2*-knockout mice and detecting aberrant alternative splicing in *CADPS2* mRNA in autistic patients. Our results suggest that defects of CADPS2 function might contribute to autism susceptibility.

Results

The mouse *Cadps2* gene was disrupted by deleting 0.9 kb of the first exon from its genomic sequence (Figure 1A), which was confirmed by Southern blot hybridization (for genomic structure, see Figure 1B) and RT-PCR (for mRNA expression, see Figure 1C). WT (*Cadps2*$^{+/+}$), heterozygous (*Cadps2*$^{+/-}$), and homozygous (*Cadps2*$^{-/-}$) pups were born at the expected 1:2:1 Mendelian frequency ($66^{+/+}$, $128^{+/-}$, and $63^{-/-}$ of 257 animals analyzed). No CADPS2 protein was detected in the cerebellum, neocortex, or hippocampus of *Cadps2*$^{-/-}$ mice, but a reduced amount was present in those of *Cadps2*$^{+/-}$ mice in relation to WT mice (Figure 1D). *Cadps2*$^{-/-}$ mice in standard breeding cages exhibited no obvious difference in life expectancy from control mice, and both male and female *Cadps2*$^{-/-}$ mice had normal reproductive ability. No significant change in appearance of *Cadps2*$^{-/-}$ mice was observed except for decreased body weight (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI29031DS1).

Nonstandard abbreviations used: BDNF, brain-derived neurotrophic factor; CADPS2, Ca^{2+}-dependent activator protein for secretion 2; DIV, days in vitro; LD, light/dark.

Conflict of interest: The authors have declared that no conflict of interest exists.

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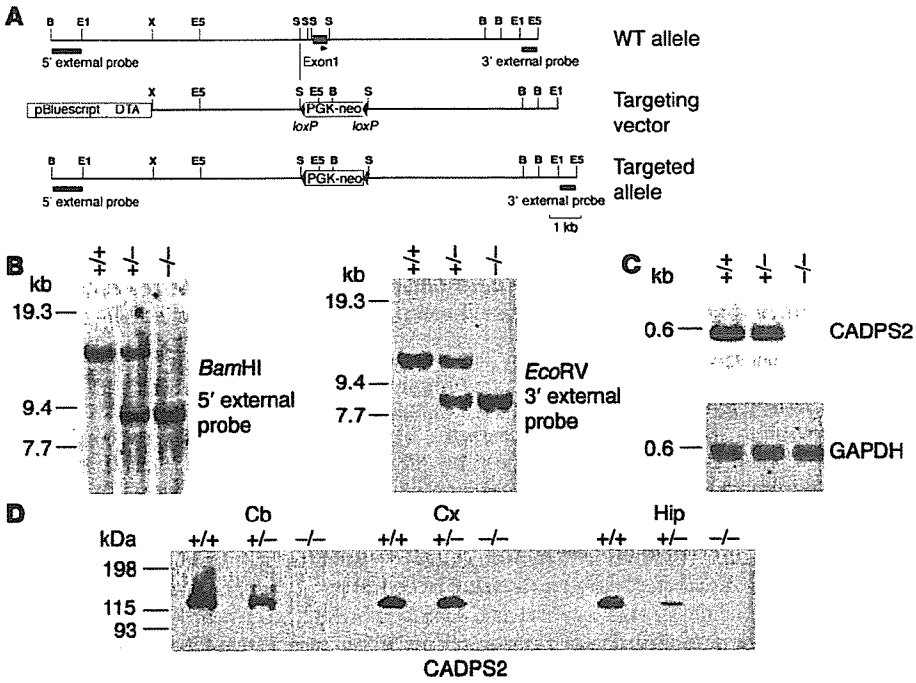

Figure 1

Generation of *Cadps2*$^{-/-}$ mice. (**A**) Maps of the *Cadps2* gene, the targeting vector, and the resultant targeted allele are shown. Probes for Southern blot analysis to screen for targeted ES clones are indicated by small solid bars. Restriction enzyme sites include the following: B, *Bam*HI; E1, *Eco*RI; E5, *Eco*RV; S, *Sma*I; X, *Xho*I. DTA, diphtheria toxin A fragment (**B**) Southern blot analysis, using the probes indicated in **A**, of DNA from tails of a single litter derived from a cross between heterozygotes. +/+, WT mice; +/–, heterozygous mice; –/–, homozygous mice. (**C**) RT-PCR banding patterns of CADPS2 using brain total RNA of P21 WT, heterozygous, and homozygous mice. The *GAPDH* gene was used as a constantly expressed control mRNA. (**D**) Immunoblot analysis of the cerebellum, neocortex, and hippocampus of P8 WT, heterozygous, and homozygous mice. Protein lysates from these brain regions were immunoblotted with anti-CADPS2 antibody. Cb, cerebellum; Cx, neocortex; Hip, hippocampus.

We first analyzed the effect of loss of CADPS2 on basal sensory and cognitive performances of *Cadps2*$^{-/-}$ mice before detailed behavioral examination. *Cadps2*$^{-/-}$ mice showed no significant difference in a visual test (visual recognition of surface to hold under tail suspension), olfactory test (scenting out hidden food), and auditory test (movements to sound stimulus) in comparison with their WT littermates (Supplemental Table 1). The Morris water maze test was performed to evaluate cognitive function of *Cadps2*$^{-/-}$ mice (Supplemental Figure 2). Escape latency to climb onto a visible platform in a pool was almost the same between WT and *Cadps2*$^{-/-}$ mice (Supplemental Figure 2A), indicating that there are no significant differences in their motor function and motivation required for this task. After 4 days of training mice to find a hidden platform, latency to reach the platform was slightly improved in *Cadps2*$^{-/-}$ mice but did not differ significantly from that in WT mice ($P = 0.106$; 2-way ANOVA with repeated measures) (Supplemental Figure 2B). By removing the platform after the last hidden platform trial, memory of the previous platform location was analyzed the next day (probe test). Interestingly, *Cadps2*$^{-/-}$ mice showed lower spatial accuracy in exploring for the platform location than WT mice ($P < 0.05$; Student's *t* test), indicating impairment in retention of spatial memory in *Cadps2*$^{-/-}$ mice (Supplemental Figure 2C).

Autism is characterized by abnormal behavioral characteristics, including impaired social interaction (1, 2), hyperactivity (17, 18), and augmented anxiety and/or reduced environmental exploration in a novel environment (1, 2, 19), and is frequently accompanied by an abnormal sleep-wake rhythm (20, 21). Detailed behavioral analyses demonstrated several autistic-like behavioral phenotypes in *Cadps2*$^{-/-}$ mice. We used male mice for the analyses of behavior but used female mice for the analysis of maternal behavior.

Firstly, the social interaction of *Cadps2*$^{-/-}$ mice was impaired. Pairs of mice of the same genotype (*Cadps2*$^{+/+}$ or *Cadps2*$^{-/-}$) that had never met were placed in a neutral cage for 20 minutes, and the frequency of interactions by *Cadps2*$^{-/-}$ mice was significantly lower than that of WT mice ($P < 0.05$; Student's *t* test; Figure 2A).

Secondly, the *Cadps2*$^{-/-}$ mice exhibited hyperactivity in their home cages. When locomotor activity was measured over a 6-day period (12-hour light/12-hour dark cycle [12-hour LD cycle]) after habituation to a fresh cage for 24 hours, the home-cage activity of the *Cadps2*$^{-/-}$ mice was significantly greater than that of their WT littermates ($P < 0.05$; Student's *t* test; Figure 2B). This difference between WT and *Cadps2*$^{-/-}$ mice was observed only in the dark cycle, during which mice are more active in general (Supplemental Figure 3).

Thirdly, the *Cadps2*$^{-/-}$ mice tended to show decreased exploratory behavior and/or increased anxiety in a novel environment. *Cadps2*$^{-/-}$ mice placed in an open field in the light cycle showed normal locomotor activity in comparison with their WT littermates, during each 5-minute block (Figure 2C). There was no significant difference between WT (13.6 ± 2.0%, mean ± SEM; $n = 17$) and *Cadps2*$^{-/-}$ mice (10.7 ± 2.0%, mean ± SEM; $n = 15$) in the time spent in a central area, which is generally accepted as a measure of fear in rodents (22). Moreover, *Cadps2*$^{-/-}$ mice showed no significant anxiety-like behavior in the LD transition test (Supplemental Figure 4, A and B). However, as shown in Figure 2D, *Cadps2*$^{-/-}$ mice became less active than WT mice during 3 time blocks (a total of 15 minutes) during which they were placed in an open field containing a novel object (the black and white vertical object shown in the inset of Figure 2D) at its center ($P < 0.01$; Student's *t* test). Representative traces from WT and *Cadps2*$^{-/-}$ mice are shown in Figures 2, E and F, respectively. The *Cadps2*$^{-/-}$ mice made significantly fewer contacts with the novel object (0.45 ± 0.10 contacts/min, mean ± SEM; $n = 15$) than the WT mice did (1.13 ± 0.20; $n = 17$) ($P < 0.01$). Another interesting finding is that in an 8-arm radial maze test, *Cadps2*$^{-/-}$ mice showed decreased locomotor

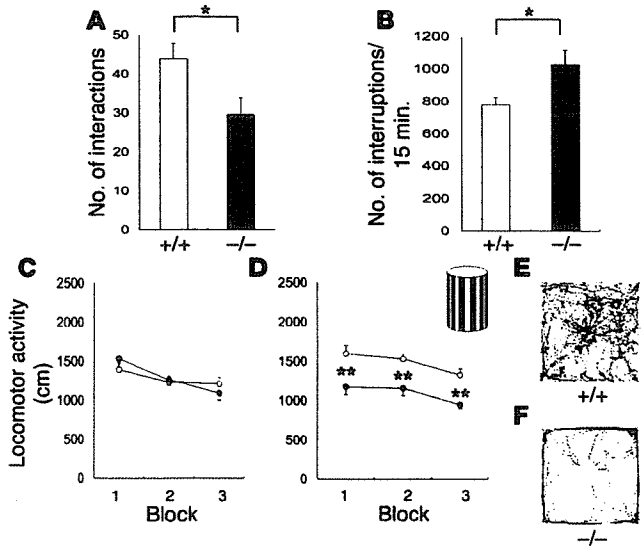

Figure 2
Autistic-like behavior of *Cadps2*$^{-/-}$ mice. (**A**) Number of reciprocal interactions of *Cadps2*$^{-/-}$ mouse pairs (black bar; $n = 12$) and of WT littermate pairs (white bar; $n = 12$) for 20 minutes. (**B**) Locomotor activity in home cages. After habituation to a fresh cage for 24 hours, the locomotor activity of *Cadps2*$^{-/-}$ mice (black bar; $n = 9$) and WT littermates (white bar; $n = 9$) was measured for 6 days (12-hour LD cycle [LD]). The number of photobeam interruptions per 15 minutes is shown in the *y* axis. (**C–F**) Horizontal locomotor activity of *Cadps2*$^{-/-}$ mice (filled circles; $n = 15$) and of WT littermates (open circles; $n = 17$) in an open field is shown in 3 blocks (5 minutes each) in the absence (**C**) or presence (**D**) of the novel object shown in the upper right of **D**. Representative movement traces of WT and *Cadps2*$^{-/-}$ are shown in **E** and **F**, respectively. Error bars indicate the SEM. $^{*}P < 0.05$; $^{**}P < 0.01$, by Student's *t* test.

activity ($P < 0.05$; Student's *t* test) and lower arm entries ($P < 0.01$; Student's *t* test) compared with those of WT mice (Supplemental Figure 5; see Discussion).

Fourthly, the *Cadps2*$^{-/-}$ mice had deficits in intrinsic sleep-wake regulation and circadian rhythmicity (Figure 3, A–C). When the circadian rhythm of locomotor activity was recorded under a 12-hour LD cycle, no difference in sleep-wake rhythm was detected between the WT and *Cadps2*$^{-/-}$ mice (Figure 3, A and B). Under constant dark (DD) conditions, the sleep-wake rhythm of the WT mice shifted to a shorter period than under LD conditions, because the internal circadian period of mice is less than 24 hours (Figure 3, A and C). The *Cadps2*$^{-/-}$ mice, however, exhibited a longer intrinsic rhythmicity under DD conditions ($P < 0.01$; Student's *t* test; Figure 3, B and C).

In addition, maternal neglect of newborns is a striking feature of *Cadps2*$^{-/-}$ mothers. Because maternal care is an important social behavior, the nurturing of newborns by their mothers was monitored. In many cages, the newborns of a *Cadps2*$^{-/-}$ dam and WT sire rarely survived beyond P1 (Figure 4A). This phenomenon was independent of litter size and almost always occurred in an all-or-none manner in each cage. At 2 days after birth, the ratio of extinction cages to survival cages was 1:14 for WT dams, 3:11 for *Cadps2*$^{+/-}$ dams, and 12:7 for *Cadps2*$^{-/-}$ dams (Figure 4B), indicating a high extinction rate for pups born from *Cadps2*$^{-/-}$ dams. In this test, heterozygote mice showed roughly intermediate phenotypes between *Cadps2*$^{-/-}$ and WT mice (Figure 4, A and B). A mildly affected level in heterozygotes was also observed in the 8-arm radial maze test (Supplemental Figure 5). These phenomena are probably a reflection of the decrease in CADPS2 protein levels in heterozygote mice (Figure 1D). We then performed cross-fostering experiments to determine whether the primary deficit resided within the *Cadps2*$^{-/-}$ dams or their pups. The results showed that pups born to control WT dams and cross-fostered to *Cadps2*$^{-/-}$ dams tended to fail to survive, whereas many pups born to *Cadps2*$^{-/-}$ dams and cross-fostered to WT dams survived (Supplemental Figure 6). These results indicate that *Cadps2*$^{-/-}$ mothers have defects in maternal behavior.

The brains of *Cadps2*$^{-/-}$ mice also exhibited several abnormal cell phenotypes. CADPS1 and CADPS2 proteins are complementarily distributed in many subregions of the mouse neocortex and hippocampus (11). At P8, CADPS2 immunoreactivity in the WT mouse neocortex was predominantly localized in pyramidal cells in cortical layer V (Figure 5A) and was colocalized with BDNF immunoreactivity (Figure 5, B and C). Underdevelopment of GABAergic interneurons is related to autism (23), and differentiation of a subset of neocortical parvalbumin-positive GABAergic neurons is regulated by BDNF (24). At P17, there were significantly fewer parvalbumin-positive interneurons in the *Cadps2*$^{-/-}$ mouse neocortex (Figure 5E) than in the WT neocortex (Figure 5, D and H), which had a similar phenotype to that of *BDNF*$^{-/-}$ mice (24). On

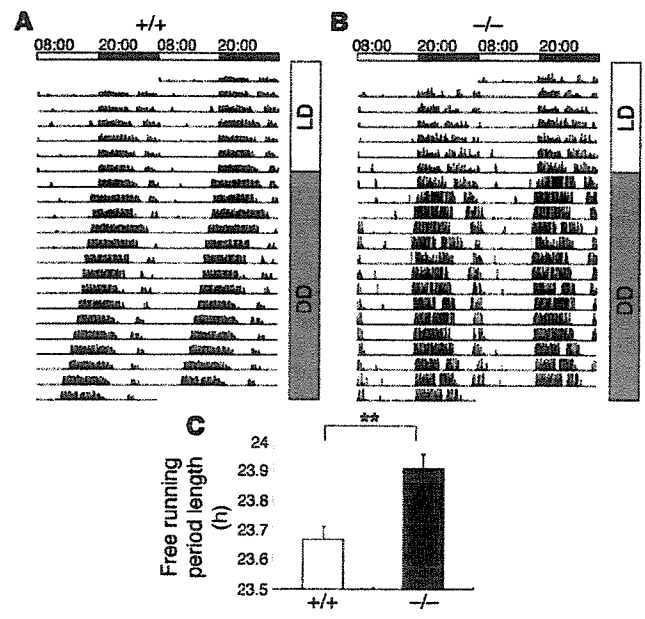

Figure 3
An abnormal sleep/wake rhythm of *Cadps2*$^{-/-}$ mice. (**A** and **B**) Sleep/wake rhythms of locomotor activity under free wheel-running conditions. Representative activity traces of WT (**A**) and *Cadps2*$^{-/-}$ (**B**) mice for 7 days of LD cycle and 14 days of constant darkness (DD) are represented as relative deflections from the horizontal line. Actograms are double-plotted over a 48-hour period. Time is expressed in a 24-hour cycle on the top of **A** and **B**. (**C**) The circadian period (h) calculated by a χ^2 periodogram of *Cadps2*$^{-/-}$ mice (black bar; $n = 9$) and WT littermates (open bar; $n = 8$). The error bars indicate the SEM. $^{**}P < 0.01$, Student's *t* test.

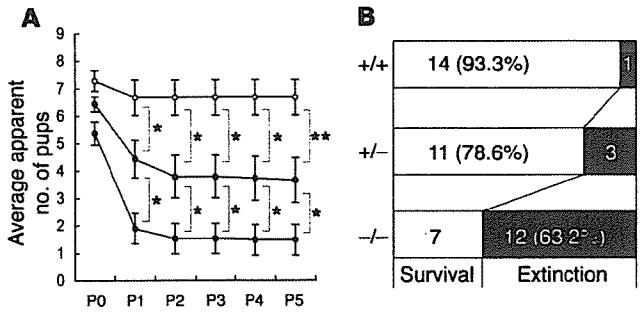

Figure 4
Maternal neglect of newborns by *Cadps2*$^{-/-}$ mothers. (**A**) Survival of pups born to virgin *Cadps2*$^{-/-}$ (closed circles; n = 19 mothers), *Cadps2*$^{+/-}$ (gray circles; n = 14 mothers), and WT (open circles; n = 15 mothers) females mated with WT males. Shown is mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$, Mann-Whitney U test. (**B**) Number of cages that contained live pups (white bars) versus number of pup extinction cages (black bars) 2 days after birth.

the other hand, there was no significant difference in the number of calbindin-positive neurons at P17 in the neocortex between WT (133 ± 15 cells/mm^2, mean ± SD) and *Cadps2*$^{-/-}$ mice (120 ± 10 cells/mm^2, mean ± SD). The decrease in the number of parvalbumin-positive interneurons in the *Cadps2*$^{-/-}$ mouse neocortex at P17 could be rescued by BDNF injection at P5 (see Methods; Figure 5, G and H), but not by vehicle injection (Figure 5, F and H). However, the number of calbindin-positive neurons was not significantly changed in *Cadps2*$^{-/-}$ mice following either PBS injection (116 ± 15 cells/mm^2, mean ± SD) or BDNF injection (119 ± 24 cells/mm^2, mean ± SD). Moreover, the levels of BDNF released into the media of *Cadps2*$^{-/-}$ mouse neocortical cell cultures at 21 days in vitro (DIV) were markedly decreased to approximately 22.1% of the levels observed in the media of WT cell cultures (Figure 5I). On the other hand, there was no significant difference in the levels of neurotrophin 3 (NT-3) or nerve growth factor between WT and *Cadps2*$^{-/-}$ culture medium at 21 DIV (NT-3: 10.3 ± 0.937 pg•mm^2/μl in WT culture versus 11.7 ± 1.078 pg•mm^2/μl in *Cadps2*$^{-/-}$ culture, mean ± SD; NGF: 395.3 ± 70.8 pg•mm^2/μl in WT culture versus 304.4 ± 71.8 pg•mm^2/μl in *Cadps2*$^{-/-}$ culture, mean ± SD).

In the WT mouse hippocampus at P8, CADPS2 immunoreactivity was localized predominantly in the stratum lucidum of hippocampal subfield CA3, where the axons of dentate granule cells (mossy fibers) are projected (Figure 6, A and D), and was colocalized with BDNF immunoreactivity (Figure 6, B, C, E, and F). Immunoreactivity of CADPS2 was not only colocalized with that of the presynaptic proteins chromogranin A and synaptophysin in the CA3 stratum lucidum but was also merged with that of Tau, an axonal marker, in hippocampal primary cultures (Supplemental Figure 7), suggesting a localization of CADPS2 in the axon terminal. By contrast, in the CA1 region, CADPS2 immunoreactive puncta were not merged with those of BDNF (Supplemental Figure 8). At P17, the *Cadps2*$^{-/-}$ hippocampus showed a reduction in the number of parvalbumin-positive interneurons in comparison with the WT hippocampus (Figure 6, G, H, and K); this reduction was also reported in *BDNF*$^{-/-}$ mice (24). The decrease in the number of parvalbumin-positive interneurons in the *Cadps2*$^{-/-}$ hippocampus at P17 could be rescued by BDNF injection at P5 to a level comparable with that of the WT hippocampus (Figure 6, J and K).

A decreased Purkinje cell survival rate is a characteristic of the cerebellum in autism (25). CADPS2 is abundant on or near vesicular structures containing BDNF in the parallel fiber terminals of presynaptic granule cells, and overexpression of exogenous CADPS2 in cerebellar primary cultures promotes the survival of postsynaptic Purkinje cells (10). There were considerably fewer calbindin-positive Purkinje cells in cell cultures of *Cadps2*$^{-/-}$ cerebellum, the number being approximately 41% of that in WT cultures (Figure 7A). However, the number of neurons positive for the neuronal differentiation marker MAP2ab, mostly granule cells, was almost the same as in WT cultures (Figure 7B), indicating that Purkinje cell viability is vulnerable to the loss of CADPS2. To verify whether increased Purkinje cell death was caused by decreased BDNF levels in *Cadps2*$^{-/-}$ cerebellar cultures, we carried

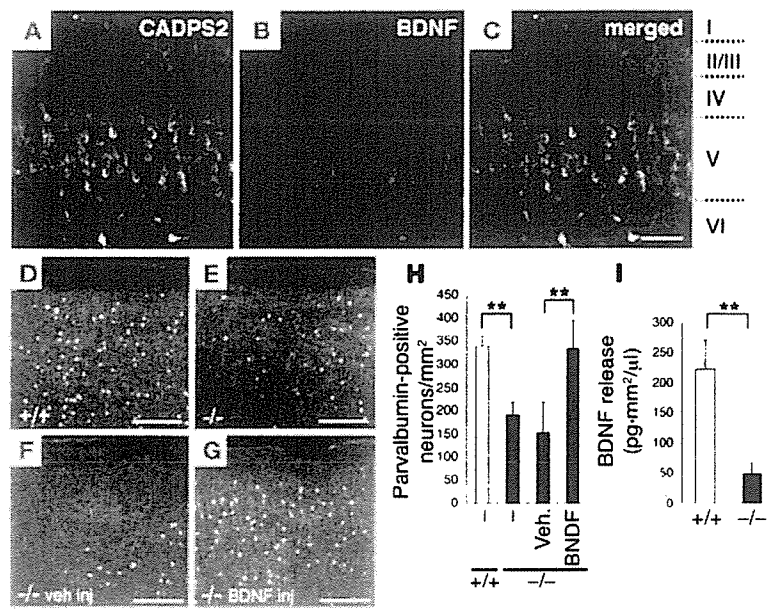

Figure 5
Abnormal immunohistochemical findings in the *Cadps2*$^{-/-}$ mouse neocortex. (**A–C**) Sagittal sections of the P8 motor cortex immunostained for CADPS2 (green in **A**) and BDNF (red in **B**). A merged image is shown in **C**. I, II/III, IV, V, and VI represent cortical layers. Scale bars: 100 μm. (**D–G**) Sagittal sections of WT (**D**) and *Cadps2*$^{-/-}$ (**E–G**) P17 motor cortex immunostained for parvalbumin. (**F** and **G**) Sections were prepared from P17 *Cadps2*$^{-/-}$ mice 12 days after an icv injection of either vehicle (veh inj) (**F**) or BDNF (BDNF inj) (**G**). Scale bars: 200 μm. (**H**) Cell density of parvalbumin-positive neurons in the P17 motor cortex. The error bars indicate SD. (**I**) BDNF release activity in WT (white bars) and *Cadps2*$^{-/-}$ (black bars) neocortical cultures was evaluated at 21 DIV by measuring the amounts of BDNF spontaneously secreted into the culture medium over the course of 21 days. Activity is indicated in BDNF concentration (pg/μl) normalized to cell density (/mm^2). Average values obtained from 6 independent experiments are shown. The error bars indicate SD. $^{**}P < 0.01$, Student's t test.

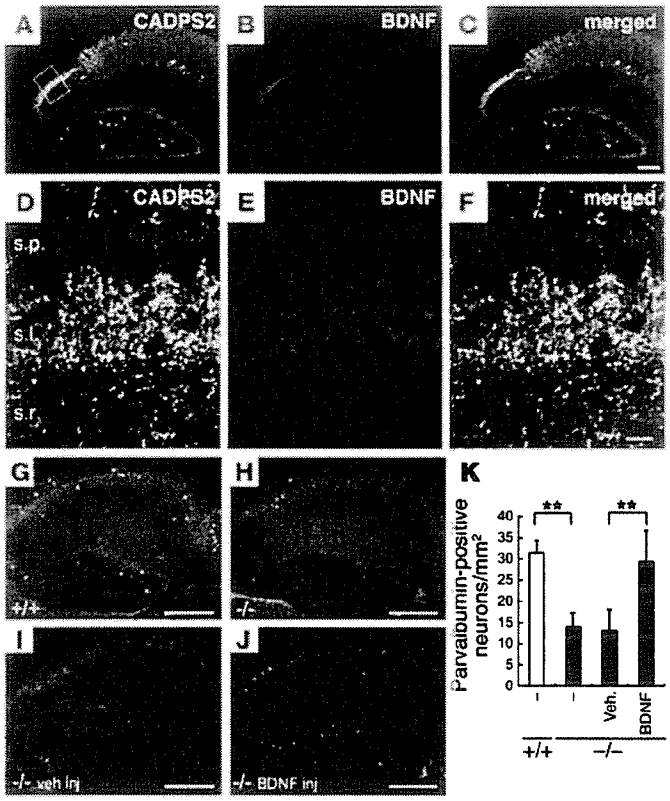

Figure 6
Abnormal immunohistochemical findings in the *Cadps2*$^{-/-}$ mouse hippocampus. (**A–C**) Sagittal sections of the P8 hippocampus immunostained for CADPS2 (green in **A**) and BDNF (red in **B**). A merged image is shown in **C**. Scale bar: 200 μm. (**D–F**) Higher magnification of the region shown by the square in **A**. s.p., stratum pyramidale; s.l., stratum lucidum; s.r., stratum radiatum. Scale bar: 10 μm. (**G–J**) Sagittal sections of WT (**G**) and *Cadps2*$^{-/-}$ (**H–J**) P17 hippocampus immunostained for parvalbumin. (**I** and **J**) Sections were prepared from P17 *Cadps2*$^{-/-}$ mice 12 days after an icv injection of either vehicle (**I**) or BDNF (**J**). Scale bars: 500 μm. (**K**) Cell density of parvalbumin-positive neurons in the P17 hippocampus. The error bars indicate SD. $^{**}P < 0.01$, Student's *t* test.

out a cell rescue assay in the presence of exogenous BDNF. By culturing cells in culture media containing BDNF (10 ng/ml), the survival rate of *Cadps2*$^{-/-}$ Purkinje cells was significantly increased in comparison with cultures containing no BDNF or heat-inactivated BDNF (at 100°C for 15 minutes, 10 ng/ml) (Figure 7A). In addition, a reduction in the number of calbindin-positive Purkinje cells was verified in sections of the P28 cerebellum (131.3 ± 7.7 cells/mm^2 in WT versus 104.4 ± 10.3 cells/mm^2 in *Cadps2*$^{-/-}$, mean ± SD; $P < 0.01$; Student's *t* test).

To investigate the role of CADPS2 in neurotrophin secretion, we examined constitutive and regulated BDNF release from primary cell cultures of *Cadps2*$^{-/-}$ mice. As shown in Figure 5I, spontaneous BDNF release from neocortical dissociated cultures at 21 DIV (constitutive release plus spontaneous activity-dependent release) was significantly reduced relative to that of WT cultures. However, BDNF was undetectable in the culture media after 50 mM KCl stimulation for 15 and 30 minutes (activity dependent, regulated release) in both WT and *Cadps2*$^{-/-}$ neocortical cultures (data not shown). Similarly, the endogenous BDNF released after KCl stimulation was hardly detected in media of cerebellar dissociated cultures at 7, 14, and 21 DIV, which was probably due to a very low amount of endogenous BDNF released from primary cultured neurons as well as insufficient sensitivity of our ELISA system, as previously described (10). We therefore exogenously overexpressed BDNF in cerebellar cultures at 5 DIV using the recombinant adenoviral vector (10). In this system, granule cells, which are the predominant cell type expressing CADPS2 in mouse cerebellum (10, 11), were preferentially infected with the adenovirus vector (26). At 48 hours after infection with the adenovirus vector, cultures expressing either BDNF-GFP (Ad-BDNF-GFP) or GFP alone (Ad-GFP) at 7 DIV were treated for 15 minutes with medium containing 10 μM NBQX (AMPA receptor inhibitor) plus 1 μM TTX (Na^+ channel inhibitor) to

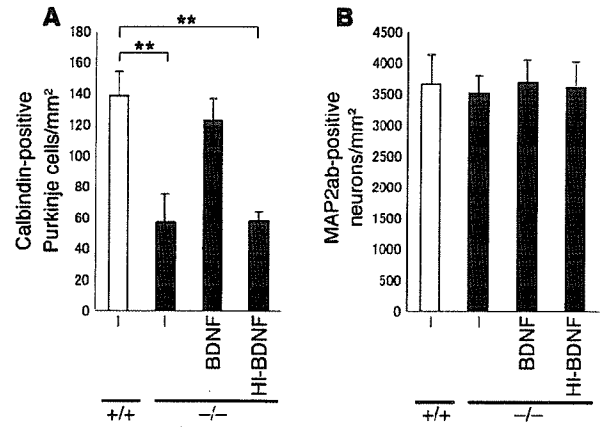

Figure 7
Increased cell death of *Cadps2*$^{-/-}$ Purkinje cells. (**A** and **B**) Cell density of calbindin-positive (**A**) and MAP2ab-positive (**B**) neurons in primary dissociated cultures (8 DIV) of WT (white bars) and *Cadps2*$^{-/-}$ (black bars) cerebella. Cerebellar cultures were grown in the absence or presence of the BDNF (10 ng/ml). Heat-inactivated BDNF (HI-BDNF; 10 ng/ml; 100°C, 15 minutes) was also used. The medium was changed at 4 DIV with readdition of BDNF or HI-BDNF. The error bars indicate SD. $^{**}P < 0.01$, Student's *t* test.

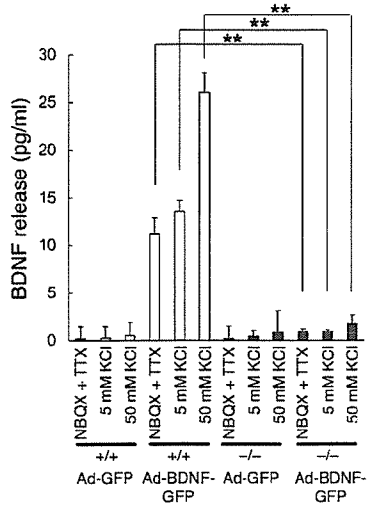

Figure 8
Decreased release of BDNF from *Cadps2*$^{-/-}$ cerebellar cultures. WT (white bars) and *Cadps2*$^{-/-}$ (black bars) cerebellar cultures were infected with the Ad-GFP and Ad-BDNF-GFP at 5 DIV. ELISA of BDNF levels released into the conditioned media from the cultures at 7 DIV with NBQX plus TTX, 5 mM KCl, and 50 mM KCl stimulation for 15 minutes. Average values obtained from 3 independent experiments are shown. There was no significant difference in MAP2ab-positive cell density and cellular BDNF contents between the WT and *Cadps2*$^{-/-}$ cultures infected with Ad-BDNF-GFP (data not shown). The error bars indicate SD. $^{**}P < 0.01$, Student's *t* test.

inhibit membrane excitability, 5 mM KCl as a control, or 50 mM KCl to induce membrane depolarization. WT cultures overexpressing exogenous BDNF showed high BDNF levels in the NBQX plus TTX and the 5 mM KCl media (mostly via constitutive release) and even higher levels of BDNF in the 50 mM KCl medium (via regulated release) compared with control (Ad-GFP) cultures (Figure 8). In contrast, such drastic increases in constitutive and regulated BDNF release levels were not observed in Ad-BDNF-GFP–infected *Cadps2*$^{-/-}$ cultures (Figure 8). These results suggest that loss of CADPS2 affects both constitutive and regulated BDNF release from cerebellar dissociated cultures.

We next examined the expression of *CADPS2* in autistic patients. Since both CADPS2 protein and CADPS1 protein are expressed in the histamine-positive basophilic leukocytes of healthy subjects (Supplemental Figure 9), we analyzed RNA samples from peripheral blood by RT-PCR with 8 primer sets that covered almost the entire CADPS2 protein-coding region. It was notable that the amplicons derived from the primer set for exons 1–5 included a shorter band (328 bp) in addition to the band of expected size (661 bp) in 3 autistic patients (Figure 9A). Moreover, only a shorter band (328 bp) was detected in 1 patient (Figure 9A). Cloning and sequencing analysis showed that the shorter, 328-bp band lacked the complete sequence of exon 3 (Figure 9C), suggesting an aberrant alternative splicing of *CADPS2* mRNA. In contrast, no exon

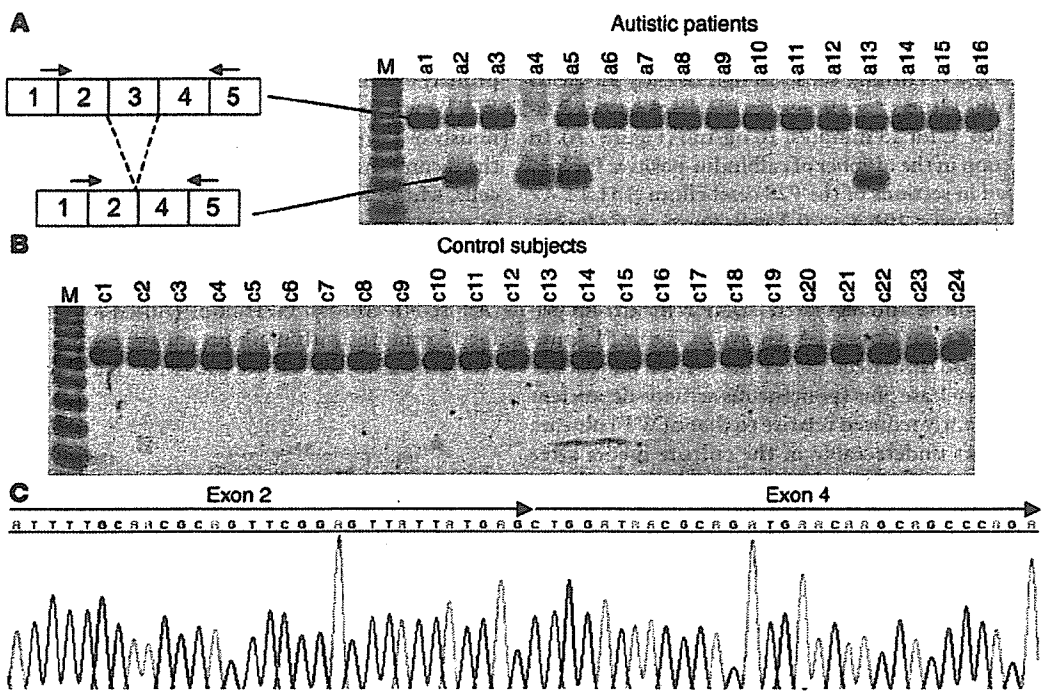

Figure 9
Aberrant *CADPS2* splicing in autistic patients. (**A** and **B**) RT-PCR analysis of *CADPS2* mRNA in blood from autistic patients (a1–a16) (**A**) and healthy control subjects (c1–c24) (**B**). A single major band (661 bp) is apparent in the control individuals. An additional band (381 bp) that resulted from skipping of exon 3 was detected in patients a2, a4, a5, and a13. The primers used are indicated on the corresponding exons by arrows. M, 100-bp ladder molecular size marker (from 200 bp at the bottom to 1000 bp at the top). (**C**) Sequencing pattern of the aberrant RT-PCR product from patient a4. The arrows indicate exon 2–exon 4 in the frame without exon 3. The same results were obtained in 3 other patients (a2, a5, and a13).

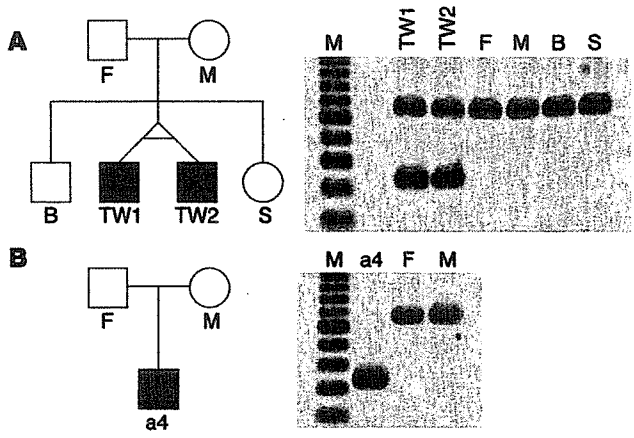

Figure 10
Expression of exon 3–skipped *CADPS2* mRNA in 2 pedigrees that include autistic patients. (**A** and **B**) Pedigree structures (left) and patterns of agarose gel electrophoresis on the RT-PCR analysis of *CADPS2* mRNA expressed in the blood of each individual (right). (**A**) Male monozygotic autistic twins (TW1 [autistic patient a5 shown in Figure 9A] and TW2 [autistic patient]) and their family members. A single major band (661 bp) produced by normal alternative splicing is apparent in their father (F); mother (M); older brother (B); and younger sister (S). On the other hand, an additional, 381-bp band produced by exon 3–skipped alternative splicing was expressed in both monozygotic autistic twins. (**B**) The autistic patient (patient a4 shown in Figure 9A), who expressed only the exon 3–skipped shorter band, and the patient's family members. Similar to the family members in **A**, a single major band of 661 bp is expressed in the father and mother. M, molecular weight marker of the 100-bp ladder (from 200 bp at the bottom to 1000 bp at the top).

3 skipping was found in any of the 24 healthy control subjects tested (Figure 9B) ($P = 0.020$; Fisher's exact probability test). Exon 3 skipping in the mouse counterpart has never been observed in the brain or nonneural tissues of WT mice (data not shown). We next examined the expression of *CADPS2* mRNA in the family members of 2 autistic patients (a4 and a5 in Figure 9A). Patient a5 in Figure 9A is a monozygotic twin, and the other autistic twin also expresses exon 3–skipped *CADPS2* mRNA (Figure 10A). However, neither their parents nor healthy family members (older brother and younger sister) were found to express the exon 3–skipped form (Figure 10A). Similarly, patient a4's family members — a father and mother who are both mentally healthy — do not express the exon 3–skipped form (Figure 10B). Although the mechanism underlying exon 3 skipping remains elusive, no mutations were found around the splice donor site (within 60 bp downstream), acceptor site (within 80 bp upstream) or branchpoint of intron 2 in the genomic sequences of these patients.

Exon 3 skipping predicts a deletion of 111 aa residues, from 119 to 229 in the human CADPS2 protein (GenBank accession number NP_060424). To investigate the effect of this splicing variant on BDNF-releasing activity, we exogenously expressed full-length (WT) or exon 3–skipped (Δexon3) mouse CADPS2 together with BDNF in PC12 cells. The BDNF released into the culture medium in response to high-KCl stimulation by cells coexpressing CADPS2(WT) was approximately 200% relative to that of cells without exogenous CADPS2 expression (Figure 11A), as described previously (10). BDNF release was also increased in cells coexpressing CADPS2(Δexon3) (Figure 11A), indicating that the exon 3–skipped CADPS2 retains BDNF-releasing activity. Moreover, in neocortical primary dissociation cell cultures of *Cadps2*$^{-/-}$ mice, the levels of BDNF released into the culture media at 21 DIV were significantly increased by transfection with either CADPS2(WT) or CADPS2(Δexon3) at 4 DIV (Figure 11B). There was no significant difference in the BDNF content of culture media between CADPS2(WT)-transfected and CADPS2(Δexon3)-transfected cultures. These results indicate that the exon 3–skipped variant has the ability to enhance release activity in in vitro culture systems.

To further investigate the function of exon 3, we screened for protein candidates that interact with the exon 3–coded region, by the yeast 2-hybrid method using exon 3–containing residue as bait (Figure 12A). As a result, we isolated 3 candidate genes, coding E1A binding protein p400 (Ep400, also known as mammalian Domino [mDomino]) (27), RAN binding protein 9 (Ranbp9, also known as RanBPM) (28), and dynactin 1 (Dctn1, also known as p150^{Glued}) (29). Proteins mDomino and RanBPM were excluded from further scrutiny because of a difference in subcellular localizations, i.e., mDomino in the nucleus (27) versus CADPS2 in the cytoplasm and membrane (30) and because of irreproducibility in coimmunoprecipitation experiments in COS-7 cells coexpressing CADPS2 and RanBPM. Two clones coding residues 951–1281 of p150^{Glued} were independently isolated. Using lysates of COS-7 cells coexpressing p150^{Glued} and CADPS2 constructs, we confirmed that the N-terminal FLAG-tagged 951–1281 residue of p150^{Glued} (40 kDa) was coimmunoprecipitated with the C-terminal HA-tagged bait region of CADPS2 (data not shown) or C-terminal HA-tagged full-length CADPS2 (Figure 12B). However, no FLAG-p150^{Glued} (951–1281 aa) was coimmunoprecipitated with exon 3–skipped CADPS2 (Figure 12B). Moreover, endogenous p150^{Glued} was coimmunoprecipitated with the endogenous CADPS2 in lysates of mouse neocortex (Figure 12C), and immunostaining signals for p150^{Glued} was merged with those for CADPS2 in primary neocortical, hippocampal, and cerebellar cultures (data not shown). These results provide strong evidence for CADPS2-p150^{Glued} interaction via the region coded by exon 3.

The protein p150^{Glued} is the most fully characterized subunit of the dynactin complex (29), which is associated with axonal transport (31, 32). We investigated the subcellular localization of exon 3–skipped CADPS2 protein in the calbindin-positive neurons of neocortical primary cultures. We used MAP2ab immunosignals to identify the axons of calbindin-positive neurons, as reported elsewhere (33). As shown in Figure 13A, expressed full-length CADPS2 protein was distributed in axons (shown as MAP2ab-negative and calbindin-positive in Figure 13C), whereas expressed exon 3–skipped CADPS2 protein was localized to dendrites and none were detected in axons (Figure 13, B and D). Similarly, full-length CADPS2 protein expressed in cerebellar primary cultures was distributed in both MAP2ab-negative axons and MAP2ab-positive dendrites (Figure 13E), whereas expressed exon 3–skipped CADPS2 protein was localized primarily to MAP2ab-positive dendrites but not to axons (Figure 13F). On the other hand, coexpression of exon 3–skipped CADPS2 and GFP-tagged BDNF in *Cadps2*$^{-/-}$ cerebellar cultures resulted in the localization of BDNF-GFP in axons, with exon 3–skipped CADPS2 being absent from axons (data not shown). This result suggests that the axonal transport of BDNF is not affected by the absence of CADPS2 protein at axons.