Table 1
Genotype and allelic distributions of the ABCA1 SNPs in patients with schizophrenia and controls.

db SNP ID and aminoacid change	Position*	Inter-SNP distance (bp)	Gender	Group	N	Genotype distribution (frequency)			$\chi 2$	P	Allele count (frequency)				$\chi 2$	P	HWE of Controls (df=1)
						R/R	*R/K*	*K/K*			*R*		*K*				
rs2230806 Arg219Lys	107620867 exon 7	(–)	All	Schizophrenia	497	119 (0.24)	241 (0.48)	137 (0.28)	2.77	0.250	479	(0.48)	515	(0.52)	0.01	0.897	$\chi^2=3.73$
				Controls	932	204 (0.22)	495 (0.53)	233 (0.25)			903	(0.48)	961	(0.52)			P=0.053
			M	Schizophrenia	274	63 (0.23)	137 (0.50)	74 (0.27)	0.47	0.789	263	(0.48)	285	(0.52)	0.45	0.503	$\chi^2=0.05$
				Controls	330	71 (0.22)	162 (0.49)	97 (0.29)			304	(0.46)	356	(0.54)			P=0.827
			F	Schizophrenia	223	56 (0.25)	104 (0.47)	63 (0.28)			216	(0.48)	230	(0.52)			$\chi^2=6.81$
				Controls	602	133 (0.22)	333 (0.55)	136 (0.23)			599	(0.50)	605	(0.50)			P=0.009
						V/V	*V/M*	*M/M*			*V*		*M*				
rs2066718 Val771Met	107589255 exon 16	31,612	All	Schizophrenia	494	438 (0.89)	54 (0.11)	2 (0.00)	1.09	0.580	930	(0.94)	58	(0.06)	0.99	0.319	$\chi^2=0.04$
				Controls	936	812 (0.87)	120 (0.13)	4 (0.00)			1744	(0.93)	128	(0.07)			P=0.847
			M	Schizophrenia	273	242 (0.89)	29 (0.11)	2 (0.01)	1.70	0.428	513	(0.94)	33	(0.06)	0.50	0.480	$\chi^2=0.30$
				Controls	333	287 (0.86)	45 (0.14)	1 (0.00)			619	(0.93)	47	(0.07)			P=0.582
			F	Schizophrenia	221	196 (0.89)	25 (0.11)	0 (0.00)	1.32	0.518	417	(0.94)	25	(0.06)	0.60	0.437	$\chi^2=0.03$
				Controls	603	525 (0.87)	75 (0.12)	3 (0.00)			1125	(0.93)	81	(0.07)			P=0.856
						I/I	*I/M*	*M/M*			*I*		*M*				
rs2066714 Ile883Met	107586753 exon 18	34,114	All	Schizophrenia	487	208 (0.43)	212 (0.44)	67 (0.14)	3.86	0.145	628	(0.64)	346	(0.36)	1.75	0.186	$\chi^2=0.91$
				Controls	917	345 (0.38)	446 (0.49)	126 (0.14)			1136	(0.62)	698	(0.38)			P=0.339
			M	Schizophrenia	266	115 (0.43)	116 (0.44)	35 (0.13)	3.23	0.199	346	(0.65)	186	(0.35)	0.87	0.335	$\chi^2=2.40$
				Controls	330	122 (0.37)	168 (0.51)	40 (0.12)			412	(0.62)	248	(0.38)			P=0.122
			F	Schizophrenia	221	93 (0.42)	96 (0.43)	32 (0.14)	1.23	0.542	282	(0.64)	160	(0.36)	0.62	0.430	$\chi^2=0.002$
				Controls	587	223 (0.38)	278 (0.47)	86 (0.15)			724	(0.62)	450	(0.38)			P=0.966
						R/R	*R/K*	*K/K*			*R*		*K*				
rs2230808 Arg1587Lys	107562804 exon 35	58,063	All	Schizophrenia	491	174 (0.35)	252 (0.51)	65 (0.13)	4.05	0.132	600	(0.61)	382	(0.39)	0.63	0.427	$\chi^2=0.50$
				Controls	923	367 (0.40)	422 (0.46)	134 (0.15)			1156	(0.63)	690	(0.37)			P=0.478
			M	Schizophrenia	273	87 (0.32)	148 (0.54)	38 (0.14)	8.51	0.014	322	(0.59)	224	(0.41)	3.68	0.055	$\chi^2=1.17$
				Controls	327	140 (0.43)	141 (0.43)	46 (0.14)			421	(0.64)	233	(0.36)			P=0.278
			F	Schizophrenia	218	87 (0.40)	104 (0.48)	27 (0.12)	0.79	0.674	278	(0.64)	158	(0.36)	0.60	0.440	$\chi^2=0.01$
				Controls	596	227 (0.38)	281 (0.47)	88 (0.15)			735	(0.62)	457	(0.38)			P=0.945

HWE; Hardy–Weinberg equilibrium.
* Chromosome position was determined from the dbSNP database.

Table 2
Genotype and allelic distributions of rs2230808 in independent replication sample.

db SNP ID and aminoacid change	Gender	Group	N	Genotype distribution (frequency)			$\chi 2$	P	Allele count (frequency)				$\chi 2$	P	HWE of controls (df=1)
				R/R	*R/K*	*K/K*			*R*		*K*				
rs2230808 Arg1587Lys	All	Schizophrenia	539	211 (0.39)	252 (0.47)	76 (0.14)	0.25	0.88	676	(0.63)	404	(0.37)	0.03	0.85	$\chi^2=0.49$
		Controls	511	201 (0.39)	233 (0.46)	77 (0.15)			635	(0.62)	387	(0.38)			P=0.48
	M	Schizophrenia	283	109 (0.39)	133 (0.47)	41 (0.14)	0.15	0.93	351	(0.62)	215	(0.38)	0.14	0.71	$\chi^2=0.01$
		Controls	267	106 (0.40)	125 (0.47)	36 (0.13)			337	(0.63)	197	(0.37)			P=0.92
	F	Schizophrenia	256	102 (0.40)	119 (0.46)	35 (0.14)	0.97	0.62	323	(0.63)	189	(0.37)	0.43	0.51	$\chi^2=1.17$
		Controls	244	95 (0.39)	108 (0.44)	41 (0.17)			298	(0.61)	190	(0.39)			P=0.28

HWE; Hardy–Weinberg equilibrium.

shown). We evaluated the difference between the all controls and all cases using ANCOVA. The female schizophrenia patients showed smaller gray matter volume in the bilateral insulae, anterior cingulate cortex, and orbitofrontal cortex, than all female controls (Fig. 1E).

We also we evaluated the difference in gray matter volume between the schizophrenic groups with and without the 1587*K* allele for each sex using ANCOVA, controlling for age, duration of illness, educational period, and medication. There were no statistically significant differences between the groups for each sex, however, male patients with schizophrenia carrying the 1587*K* allele showed small gray matter volume in the left occipital region and bilateral posterior cingulate cortices, almost the same as Figure (B), compared with those who did not carry the 1587*K* allele at nominal trend level (F) ($P<0.01$ uncorrected). There were no differences between the female schizophrenic patients with or without the 1587*K* allele using loose criteria ($P<0.01$ uncorrected, data not shown).

4. Discussion

We found that the 1587*K* allele of *ABCA1* was significantly more common in male patients with schizophrenia than in male controls. However, such a difference was not observed in women. Furthermore, our results showed that male schizophrenic patients who carried the 1587*K* allele have smaller gray matter volume than in those who did not, but this difference did not extend to women. To our knowledge, this is the first study that reports the possible association of *ABCA1* with susceptibility to schizophrenia and related brain abnormalities.

4.1. ABCA1 polymorphisms and susceptibility to schizophrenia

The 1587*K* allele was reported to increase cerebrospinal fluid tau level and brain amyloid beta load (Katzov et al., 2004). It was also associated with low plasma levels of apolipoprotein A1 (Tregouet et al., 2004) and HDL-cholesterol (Clee et al., 2001; Frikke-Schmidt et al., 2004), suggesting functional differences between the *R*1587 and 1587*K* alleles, which may explain our results.

The present study showed gender-specific association between *R*1587*K* (rs2230808) and schizophrenia in our population. Serum from men displays an enhanced free cholesterol efflux capacity via the ABCA1 transporter pathway compared with that from perimenopausal women (Catalano et al., 2008). Estradiol was known to modulate a wide range of functions of the brain. From the onset of menopause, declining levels of estradiol can cause cognitive disturbances and changes in behavior that can be counterbalanced by hormone replacement. Studies in mice have suggested that the atheroprotective effects of estrogen may occur partly via the ABCA1-mediated pathway (Srivastava, 2002). Another study found that ABCA1 was up-regulated by estradiol (Sárvári et al., 2010). Taking these previous findings into consideration, the observed sex difference in our study may be explained, at least in part, by the fact that estrogen is involved in the regulation of ABCA1 activity. The role of CNS cholesterol in synaptic function and neurodegenerative disorders has recently been appreciated, but the mechanisms regulating its transport and homeostasis are only partially understood. Therefore, further studies that focused on the sex difference should be needed to reveal the function of the ABCA1.

In the initial study, the 1587*K* allele (rs2230808) was significantly more common in male patients with schizophrenia than in male controls. Although such a significant difference was not observed in the second sample alone, the increased frequency of the 1587K allele in male patients remained to be significant in the combined male sample. Though there was the association of *ABCA1* with susceptibility to schizophrenia, it is suggested that this relationship may be fairly weak.

4.2. ABCA1 polymorphism and MRI volumetry

Our results showed that male schizophrenia patients carrying the 1587*K* allele showed smaller gray matter volume than those who did not carry the allele. Schizophrenia has been associated with volume reductions in the limbic, paralimbic, frontal, and temporal cortical regions (Glahn et al., 2008; Ellison-Wright et al., 2008; Shenton et al., 2001; Wright et al., 2000), although some previous studies did not detect disturbances in such regions (Kanaan et al., 2005; Kubicki et al.,

Table 3
Characteristics of the subjects who underwent MRI.

		Genotype distribution of rs2230808	N	Age	Duration of illness	Drug dose (chlorpromazine equivalent)
Men	Control	*R/R*	21	39.8±12.4 (20–71)		
		K carrier	26	38.9±11.9 (25–69)		
		All	47	39.3±12.0 (20–71)		
	Schizophrenia	*R/R*	16	44.7±16.7 (22–76)	21.0±16.9	770.0±636.0
		K carrier	33	43.7±13.0 (27–72)	21.7±12.9	1183.6±945.7
Women	Control	*R/R*	39	48.2±13.5 (25–74)		
		K carrier	53	39.9±11.6 (22–71)		
		All	92	43.4±13.0 (22–74)		
	Schizophrenia	*R/R*	16	47.5±12.3 (22–67)	17.2±13.2	691.6±566.3
		K carrier	21	46.3±15.0 (23–75)	19.0±13.2	731.2±623.9

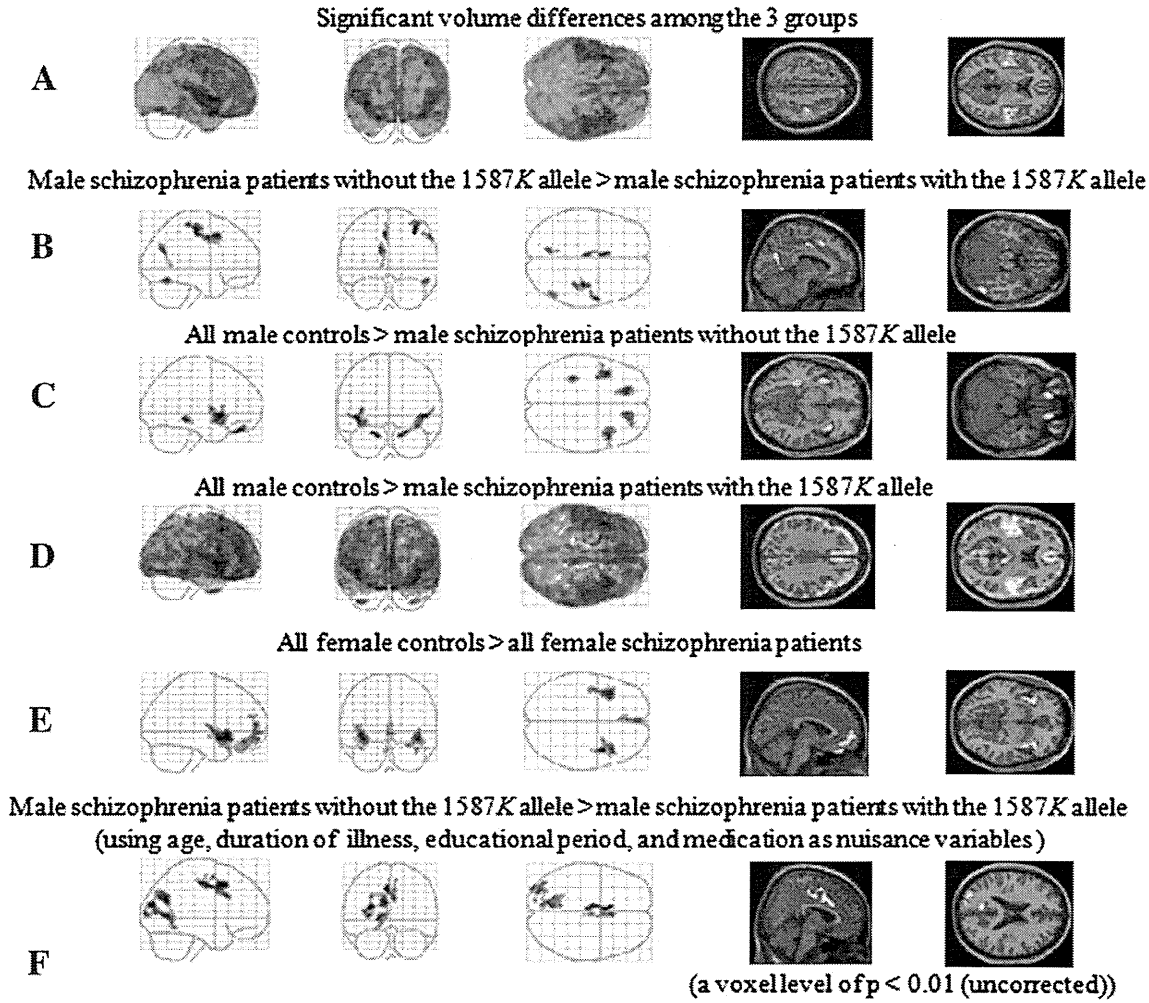

Fig. 1. Group effect was assessed using analysis of covariance (ANCOVA) (SPM2). Age was used as a nuisance variable. (A); There were statistically significant volume differences among the 3 groups of men, i.e., the male schizophrenia patients with and without the 1587*K* allele and the entire male control group. (B); Male schizophrenia patients carrying the 1587*K* allele showed gray matter volume reduction in the bilateral occipital regions and posterior cingulate cortices compared with those who did not carry this allele. (C); There were volume decreases in the bilateral insulae and orbitofrontal regions, and the left parahippocampal region in male patients with schizophrenia without the 1587*K* allele compared with all male controls. (D); Male patients with schizophrenia carrying the 1587*K* allele showed volume reduction in almost all the gray matter areas, compared with all male controls. (E); When all female schizophrenia patients were analyzed collectively, they showed gray matter volume reduction in the bilateral insulae, anterior cingulate cortex, and orbitofrontal cortex, compared with all female controls. (F); We also evaluated the difference in gray matter volume between the schizophrenic groups with and without the 1587*K* allele for each sex using ANCOVA, controlling for age, duration of illness, educational period, and medication. Male patients with schizophrenia carrying the 1587*K* allele showed small gray matter volume in the left occipital region and bilateral posterior cingulate cortices, compared with those who did not carry the 1587*K* allele controlling for age, duration of illness, educational period, and medication, at nominal trend level ($P<0.01$ uncorrected).

2007). Two broad theories have been proposed to describe the pattern of cerebral changes: the global and macro-circuit theories (Buchsbaum et al., 2006). According to the global theory, white matter reductions occur uniformly throughout the brain, possibly as a result of genetic abnormalities in the protein pathways controlling myelination (Konrad and Winterer, 2008). The alternative macro-circuit theory proposes that specific white matter tracts are disrupted in schizophrenia either as a cause or a consequence of a disorder in the gray matter regions they connect (Konrad and Winterer, 2008). The present results may accord with the global theory by showing smaller volume in almost the entire gray matter in male schizophrenia patients carrying the 1587*K* allele of *ABCA1*, because ABCA1 was regarded as the key regulator of brain cholesterol homeostasis and associated with structure and function in neurons such as myelination (Karasinska et al., 2009). Both male and female schizophrenia patients who did not carry the 1587*K* allele showed smaller volume in the medial temporal region, insulae, and anterior cingulate cortex, which have been referred to as predominantly impaired brain regions in schizophrenia, than control subjects (Glahn et al., 2008; Ellison-Wright et al., 2008). On the other hand, the male patients with schizophrenia carrying the 1587*K* allele showed the smaller volume in the occipital regions and posterior cingulate cortices, where it is known to remain unchanged from illness, than male patients not carrying 1587*K* allele. Intricate analysis controlling for age, duration of illness, educational period, and medication, male patients carrying 1587*K* allele showed the smaller volume in occipital and posterior cingulate cortices compared with male patients not carrying 1587*K* allele, only at the trend level, but these tendencies could not be detected in females even at the trend level. From these points, we suggest a male-specific association of the 1587*K* allele of *ABCA1* with susceptibility to schizophrenia and smaller gray matter volume in schizophrenia. In this study, we evaluated only a gray matter volume change, and no consideration was paid to the white matter. Further work with the diffusion tensor imaging data will be necessary to confirm our results.

Schizophrenia is a multifactorial disorder caused by a complex interaction of genetic and environmental factors (Bassett et al., 2001).

In this study, we found no significant difference in gray matter volume related to the *R1587K* polymorphism in healthy subjects. This may be accounted for by the possibility that *ABCA1* polymorphism interacts with other risk factors for schizophrenia and that these collectively influence brain vulnerability.

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Possible association between *Interleukin-1beta* gene and schizophrenia in a Japanese population

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Abstract

Background: Several lines of evidence have implicated the pro-inflammatory cytokine interleukin-1beta (IL-1β) in the etiology of schizophrenia. Although a number of genetic association studies have been reported, very few have systematically examined gene-wide tagging polymorphisms.

Methods: A total of 533 patients with schizophrenia (302 males: mean age ± standard deviation 43.4 ± 13.0 years; 233 females; mean age 44.8 ± 15.3 years) and 1136 healthy controls (388 males: mean age 44.6 ± 17.3 years; 748 females; 46.3 ± 15.6 years) were recruited for this study. All subjects were biologically unrelated Japanese individuals. Five tagging polymorphisms of *IL-1β* gene (rs2853550, rs1143634, rs1143633, rs1143630, rs16944) were examined for association with schizophrenia.

Results: Significant difference in allele distribution was found between patients with schizophrenia and controls for rs1143633 ($P = 0.0089$). When the analysis was performed separately in each gender, significant difference between patients and controls in allele distribution of rs1143633 was observed in females ($P = 0.0073$). A trend towards association was also found between rs16944 and female patients with schizophrenia ($P = 0.032$).

Conclusions: The present study shows the first evidence that the *IL-1β* gene polymorphism rs1143633 is associated with schizophrenia susceptibility in a Japanese population. The results suggest the possibility that the influence of *IL-1β* gene variations on susceptibility to schizophrenia may be greater in females than in males. Findings of the present study provide further support for the role of IL-1β in the etiology of schizophrenia.

Background

Several lines of evidence suggest that pro-inflammatory cytokine interleukin-1beta (IL-1β) is implicated in the etiology and pathophysiology of schizophrenia. Although studies investigating peripheral levels of IL-1β in schizophrenic patients have reported inconsistent results [1-6], a study examining the cerebrospinal fluid has shown a marked elevation of IL-1β in patients with first-episode schizophrenia compared to healthy controls [7]. Kowalski et al [8] reported that the release of IL-1β by peripheral monocytes was increased before treatment and then normalized by antipsychotic medication in patients with schizophrenia. Recently, Liu et al. [9] showed that IL-1β in the peripheral blood mononuclear cells was overexpressed not only in schizophrenia patients but also in their siblings, suggesting the involvement of the hereditary factors. Furthermore, previous findings suggested that IL-1β may be involved in the possible link between prenatal exposure to infection and schizophrenia [10,11].

The *IL-1β* gene is located in a region on 2q14. This region has consistently shown positive linkage findings in schizophrenia. Many studies have reported this region among their largest results [12,13]. Furthermore, Lewis et al [14] have shown in their meta-analysis of 20 genome scans that 2p12-q22.1 was associated with a genomewide significant *P* value. Linkage of this region with schizophrenia in an Asian population has also been reported [15].

A number of genetic association studies have suggested that genetic variation of the *IL-1β* gene might confer susceptibility to schizophrenia. Three studies in Caucasian populations reported a significant association of schizophrenia with an *IL-1β* gene polymorphism

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rs16944 [16-18]. However, this association was not confirmed in other studies [19,20]. Furthermore, none of the previous studies in Asian populations have obtained evidence for an association between *IL-1β* gene and schizophrenia [21-23]. All of the aforementioned association studies, except for that of Shirts, et al. [19], examined only rs16944 and/or rs1143634. Therefore, the role of other *IL-1β* gene polymorphisms remains to be determined. We here examined 5 tagging polymorphisms of the *IL-1β* gene for an association with schizophrenia in a Japanese sample.

Methods

Subjects

Subjects were 533 patients with schizophrenia (302 males: mean age ± standard deviation 43.4 ± 13.0 years; 233 females; mean age 44.8 ± 15.3 years) and 1136 healthy controls (388 males: mean age 44.6 ± 17.3 years; 748 females; 46.3 ± 15.6 years). The mean age at onset was 23.9 ± 8.0 and 25.8 ± 9.8 years for male and female patients, respectively. All subjects were biologically unrelated Japanese individuals, based on their self-reports, and were recruited from the outpatient clinic of the National Center of Neurology and Psychiatry Hospital, Tokyo, Japan or through advertisements in free local information magazines and by our website announcement. Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4^{th} edition criteria [24], on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers with no current or past history of psychiatric treatment, and were screened using the Japanese version of the Mini International Neuropsychiatric Interview (M.I.N.I.) [25,26] by a research psychiatrist to rule out any axis I psychiatric disorders. 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. The study protocol was approved by the ethics committee at the National Center of Neurology and Psychiatry, Japan. After description of the study, written informed consent was obtained from every subject. Most of the subjects had participated in our previous genetic association studies [27,28]. Some of the control subjects had also participated in our previous studies which examined *IL-1β* gene polymorphisms [29,30].

Genotyping

Five tagging single nucleotide polymorphisms (SNPs) (rs2853550, rs1143634, rs1143633, rs1143630, rs16944) in a region 1 kilobase (kb) upstream to 1 kb downstream of the *IL-1β* gene (chromosome 2: 113,302,808 - 113,311,827 bp) were selected by Haploview 4.2 [31] using Japanese and Chinese population in the HapMap SNP set (version 22), at an r^2 threshold of 0.80 with a minor allele frequency greater than 0.1. Genomic DNA was prepared from the venous blood according to standard procedures. The SNPs were genotyped using the TaqMan 5'-exonuclease allelic discrimination assay. Thermal cycling conditions for polymerase chain reaction were 1 cycle at 95°C for 10 minutes followed by 50 cycles of 92°C for 15 seconds and 60°C for 1 minute. The allele-specific fluorescence was measured with ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems, Foster city, CA, USA). Genotype data were read blind to the case-control status. Ambiguous genotype data were not included in the analysis. The call rates for each SNP ranged from 97.7% to 98.6%. The genotyping failure rate for all SNPs combined was < 2%. In 92 subjects, all 5 SNPs were genotyped in duplicate to ensure genotyping accuracy, and the concordance rate of called genotypes was over 99%.

Statistical analysis

Deviations of genotype distributions from the Hardy-Weinberg equilibrium (HWE) were assessed with the exact test described by Wigginton et al [32]. Genotype and allele distributions were compared between patients and controls by using the χ^2 test for independence or with Fisher's exact test. The above statistical analyses were performed using PLINK version 1.07 [33].

Haploview 4.2 [31] was used to estimate haplotype frequencies and linkage disequilibrium (LD) coefficients. Haplotypes with frequencies > 1% were included in the association analysis. Permutation procedure (10,000 replications) was used to determine the empirical significance.

Statistical tests were two tailed and statistical significance was considered when $P < 0.05$. Significance level corrected for multiple comparisons of 5 SNPs was set at $P < 0.013$ by a method proposed by Li et al [34], which was calculated using SNPSpD (SNP Spectral Decomposition) software [35].

Power calculations were performed using the Power Calculator for Two Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/). Power was calculated under prevalence of 0.01 using an allelic model with an alpha level of 0.05. Assuming disease allele frequencies of 0.20 and 0.40, our sample had 80% statistical power to detect relative risks of 1.28 and 1.23, respectively. Similarly, we had 90% power to detect relative risks of 1.33 and 1.27.

Since several aspects of immunity have marked sex differences [36], analyses were performed not only for the entire sample but also for each gender separately. Assuming allele frequency of 0.40, male and female samples each had 80% statistical power to detect relative risks of 1.35 and 1.34, respectively.

Table 1 Association analysis of the 5 SNPs in both genders combined

SNP name	Allele 1/2		Males							
			N	Genotype			Allele		P-value		HWE P-value
				1/1	1/2	2/2	1	2	Genotype	Allele	
rs2853550	A/G	Schizophrenia	531	9	128	394	146	916	0.23	0.088	0.86
				(0.02)	(0.24)	(0.74)	(0.14)	(0.86)			
		Controls	1115	14	232	869	260	1970			0.88
				(0.01)	(0.21)	(0.78)	(0.12)	(0.88)			
rs1143634	A/G	Schizophrenia	525	1	41	483	43	1007	0.97[(a)]	0.90	0.59
				(0.00)	(0.08)	(0.92)	(0.04)	(0.96)			
		Controls	1121	2	90	1029	94	2148	1.00		
				(0.00)	(0.08)	(0.92)	(0.04)	(0.96)			
rs1143633	C/T	Schizophrenia	524	111	249	164	471	577	0.035	0.0089	0.38
				(0.21)	(0.48)	(0.31)	(0.45)	(0.55)			
		Controls	1123	188	525	410	901	1345			0.38
				(0.17)	(0.47)	(0.37)	(0.40)	(0.60)			
rs1143630	T/G	Schizophrenia	520	13	140	367	166	874	0.88	0.66	1.00
				(0.03)	(0.27)	(0.71)	(0.16)	(0.84)			
		Controls	1119	24	296	799	344	1894			0.65
				(0.02)	(0.26)	(0.71)	(0.15)	(0.85)			
rs16944	A/G	Schizophrenia	521	123	253	145	499	543	0.18	0.060	0.54
				(0.24)	(0.49)	(0.28)	(0.48)	(0.52)			
		Controls	1111	226	534	351	986	1236			0.39
				(0.20)	(0.48)	(0.32)	(0.44)	(0.56)			

(a) Calculated using Fisher's exact test.

SNP: single nucleotide polymorphism; HWE: Hardy-Weinberg Disequilibrium

Numbers in parentheses represent the frequencies of genotypes and alleles.

Results

Genotype and allele distributions of the examined SNPs for the entire sample, males, and females are shown in Table 1, 2, and 3, respectively. The genotype distributions did not significantly deviate from the HWE in any of the SNPs examined. Significant differences in genotype and allele distributions were found between the patients with schizophrenia and controls for rs1143633. The C allele was significantly more common in patients than in controls (odds ratio 1.22, 95% confidence interval (CI) 1.05 to 1.41, $P = 0.0089$). This association remained significant after correcting for multiple testing of 5 SNPs (corrected $P = 0.013$). When the analysis was performed separately in each gender, significant difference between patients and controls in allele distribution of rs1143633 was observed only in females (odds ratio 1.34, 95% CI 1.08 to 1.66, $P = 0.0073$). The A allele of rs16944 also showed a trend towards association with schizophrenia in female subjects (odds ratio 1.26, 95% CI 1.02 to 1.56, $P = 0.032$).

Linkage disequilibrium (LD) coefficients (D' and r^2) and haplotype blocks are shown in Figure 1. Results of the haplotype association analyses are shown in Table 4. No significant difference in haplotype distribution was found between patients with schizophrenia and controls (all $P > 0.05$ by permutation test).

Discussion

To our knowledge, the present study is the largest study to date that examined the *IL-1β* gene polymorphisms for association with schizophrenia. The results provide the first evidence suggesting that the C allele of rs1143633 is associated with schizophrenia.

The study in a United States population by Shirts et al [19] was the only one that previously examined the association of schizophrenia with rs1143633, in which no significant difference was found in allele frequencies between patients and controls. Although Watanabe et al [23] have also examined 9 SNPs of the IL-1 gene complex in Japanese subjects, none of the SNPs examined in their study was in remarkable linkage disequilibrium with rs1143633 or rs16944 (all $r^2 < 0.1$ based on HapMap Japanese and Han Chinese population data, release 22). The inconsistent results regarding the effect of rs1143633 between Shirts, et al [19] and our study may be attributable to ethnic difference. Indeed, a recent meta-analysis has shown a significant association of the G allele of rs16944 and the G allele carrier status of rs1143634 with a risk of schizophrenia in Caucasian, but not in Asian, populations [37]. Our samples provided sufficient power to detect relatively small relative risks, and therefore suggest that rs16944 and rs1143634 have no major effect on

Table 2 Association analysis of the 5 SNPs in males

SNP name	Allele 1/2		Males								
			N	Genotype			Allele		P-value		HWE P-value
				1/1	1/2	2/2	1	2	Genotype	Allele	
rs2853550	A/G	Schizophrenia	300	4	74	222	82	518	0.68[(a)]	0.69	0.62
				(0.01)	(0.25)	(0.74)	(0.14)	(0.86)			
		Controls	383	7	85	291	99	667			0.82
				(0.02)	(0.22)	(0.76)	(0.13)	(0.87)			
rs1143634	A/G	Schizophrenia	298	0	24	274	24	572	0.81[(a)]	0.82	1.00
				(0.00)	(0.08)	(0.92)	(0.04)	(0.96)			
		Controls	383	1	27	355	29	737			0.42
				(0.00)	(0.07)	(0.93)	(0.04)	(0.96)			
rs1143633	C/T	Schizophrenia	299	59	145	95	263	335	0.43	0.47	0.81
				(0.20)	(0.48)	(0.32)	(0.44)	(0.56)			
		Controls	383	77	168	138	322	444			0.059
				(0.20)	(0.44)	(0.36)	(0.42)	(0.58)			
rs1143630	T/G	Schizophrenia	295	7	81	207	95	495	0.75	0.73	1.00
				(0.02)	(0.27)	(0.70)	(0.16)	(0.84)			
		Controls	383	6	106	271	118	648			0.32
				(0.02)	(0.28)	(0.71)	(0.15)	(0.85)			
rs16944	A/G	Schizophrenia	295	66	143	86	275	315	0.92	0.67	0.64
				(0.22)	(0.48)	(0.29)	(0.47)	(0.53)			
		Controls	385	82	186	117	350	420			0.61
				(0.21)	(0.48)	(0.30)	(0.45)	(0.55)			

(a) Calculated using Fisher's exact test.

SNP: single nucleotide polymorphism; HWE: Hardy-Weinberg Disequilibrium

Numbers in parentheses represent the frequencies of genotypes and alleles.

Table 3 Association analysis of the 5 SNPs in females

SNP name	Allele 1/2		Males								
			N	Genotype			Allele		P-value		HWE P-value
				1/1	1/2	2/2	1	2	Genotype	Allele	
rs2853550	A/G	Schizophrenia	231	5	54	172	64	398	0.18	0.096	0.78
				(0.02)	(0.23)	(0.74)	(0.14)	(0.86)			
		Controls	732		7	147	578		161	1303	0.57
				(0.01)	(0.20)	(0.79)	(0.11)	(0.89)			
rs1143634	A/G	Schizophrenia	227	1	17	209	19	435	0.46[(a)]	0.84	0.32
				(0.00)	(0.07)	(0.92)	(0.04)	(0.96)			
		Controls	738	1	63	674	65	1411			1.00
			(0.00)	(0.09)	(0.91)	(0.04)	(0.96)				
rs1143633	C/T	Schizophrenia	225	52	104	69	208	242	0.013	**0.0073**	0.29
			(0.23)	(0.46)	(0.31)	(0.46)	(0.54)				
		Controls	740	111	357	272	579	901			0.76
			(0.15)	(0.48)	(0.37)	(0.39)	(0.61)				
rs1143630	T/G	Schizophrenia	225	6	59	160	71	379	0.97	0.83	0.80
			(0.03)	(0.26)	(0.71)	(0.16)	(0.84)				
		Controls	736	18	190	528	226	1246			0.89
			(0.02)	(0.26)	(0.72)	(0.15)	(0.85)				
rs16944	A/G	Schizophrenia	226	57	110	59	224	228	0.11	0.032	0.69
			(0.25)	(0.49)	(0.26)	(0.50)	(0.50)				
		Controls	726	144	348	234	636	816			0.50
			(0.20)	(0.48)	(0.32)	(0.44)	(0.56)				

(a) Calculated using Fisher's exact test.

SNP: single nucleotide polymorphism; HWE: Hardy-Weinberg Disequilibrium

Numbers in parentheses represent the frequencies of genotypes and alleles. Significant P-values (< 0.013) are shown in boldface.

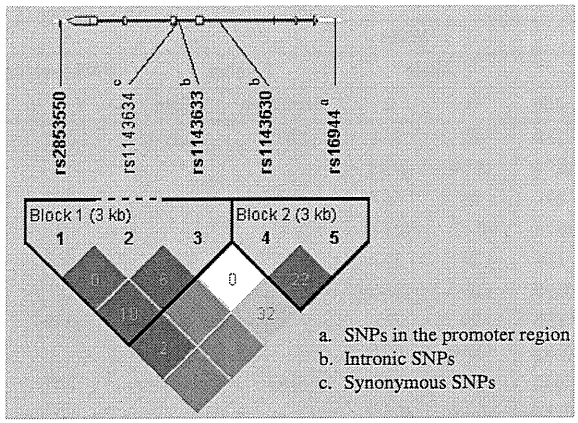

Figure 1 Haplotype block structure of the *IL-1β* gene. Genomic organization and linkage disequilibrium (LD) structure of the *IL-1β* gene are shown. Exons are shown as boxes. Shades of red represent extent of LD (darker red denotes D' = 1). Numbers in squares give r^2 values multiplied by 100.

schizophrenia susceptibility in Asian populations, which is consistent with the previous Asian findings [21-23]. However, there was a trend of association of rs16944, in the opposite direction to that of the Caucasians, with schizophrenia susceptibility in female subjects. Therefore, there remains a possibility that a larger study would yield a significant difference between Japanese female schizophrenic patients and controls in the allele frequency of rs16944.

A number of genome-wide association studies (GWAS) have searched for polymorphisms associated with schizophrenia [38-43]. Although no evidence of association with *IL-1β* gene has been reported, common risk alleles in the major histocompatibility region on chromosome 6, which is involved in the immune response, have shown statistically significant evidence of association [38-40]. Furthermore, a genome-wide pharmacogenomic study has shown that *IL-1α* rs11677416, which is in weak LD with rs1143633 (r^2 = 0.094, D' = 0.809 based on HapMap Japanese and Han Chinese population data, release 22), was associated with response of neurocognitive symptoms to antipsychotic treatment [44]. These findings, together with ours, suggest genetic influence on immune alterations in schizophrenia.

A shift towards the T helper type 2 (Th2) system has been indicated in schizophrenia [45-47]. IL-1β stimulates

Table 4 Haplotype analysis of *IL-1β* gene polymorphisms

			Males					Females				
Block	Haplotype	Diagnosis	Carrier	Non-carrier	χ2	Nominal P value	Permutation P value	Carrier	Non-carrier	χ2	Nominal P value	Permutation P value
	GT	Schizophrenia	336.3	265.7	0.557	0.456	0.957	251.0	213.0	6.240	0.0125	0.118
			(0.559)	(0.441)				(0.541)	(0.459)			
		Controls	447.9	326.1				901.0	585.0			
			(0.579)	(0.421)				(0.606)	(0.394)			
1	GC	Schizophrenia	183.1	418.9	0.216	0.642	0.995	149.0	315.0	2.298	0.130	0.691
			(0.304)	(0.696)				(0.321)	(0.679)			
		Controls	226.4	547.6				422.5	1063.5			
			(0.293)	(0.707)				(0.284)	(0.716)			
	AC	Schizophrenia	82.6	519.4	0.215	0.643	0.995	63.7	400.3	3.281	0.0701	0.461
			(0.137)	(0.863)				(0.137)	(0.863)			
		Controls	99.6	674.4				158.5	1327.5			
			(0.129)	(0.871)				(0.107)	(0.893)			
	GG	Schizophrenia	321.4	280.6	0.154	0.694	0.996	231.2	228.8	5.012	0.0252	0.207
			(0.534)	(0.466)				(0.503)	(0.497)			
		Controls	422.6	353.4				837.4	652.6			
			(0.545)	(0.455)				(0.562)	(0.438)			
2	GA	Schizophrenia	183.5	418.5	0.040	0.841	1.00	156.4	303.6	5.326	0.0210	0.178
			(0.305)	(0.695)				(0.340)	(0.660)			
		Controls	232.7	543.3				422.8	1067.2			
			(0.300)	(0.700)				(0.284)	(0.716)			
	TA	Schizophrenia	97.1	504.9	0.081	0.776	0.999	72.4	387.6	0.027	0.869	1.00
			(0.161)	(0.839)				(0.157)	(0.843)			
		Controls	120.7	655.3				229.8	1260.2			
			(0.156)	(0.844)				(0.154)	(0.846)			

Numbers in parentheses represent the frequencies of haplotypes. Permutation P values were based on 10,000 permutations.

the production of prostaglandin E2, which is an important cofactor for the induction of T-helper lymphocyte activity towards Th2 direction. Significant increase in circulating mRNA expression levels of IL-1β has been observed in schizophrenic patients [9]. The changes in mRNA levels may reflect the genetic variation in *IL-1β* gene. The findings on biological roles of *IL-1β* polymorphisms, however, have not been consistent across studies. A/A genotype of rs16944 has been associated with higher gastric mucosa IL-1β levels in H. pylori positive population [48]. On the other hand, subjects with G/G genotype showed an increased release of IL-1β from mononuclear cells after stimulation with lipopolysaccharide [49]. Recent studies suggest that the functional role of rs16944 may depend on the *IL-1β* promoter region haplotypes including rs16944 and rs1143627 [50-53]. Although the findings are inconsistent, these previous studies suggest that rs16944 could affect the expression levels of IL-1β. On the other hand, the influence of rs1143633 on IL-1β expression levels has not been previously reported.

Intriguingly, rs1143633 and rs16944 have also been associated with cortisol response to dexamethasone in healthy subjects [30]. Alleles associated with increased cortisol response to dexamethasone were shown to be associated with schizophrenia in the present study. Higher rates of non-suppression to dexamethasone compared to healthy subjects have been reported in schizophrenia [54] and schizotypy [55]. On the other hand, Ismail et al [56] reported that less than 2% of their schizophrenic patients were non-suppressors. Although the findings are inconsistent, these studies indicate that schizophrenia may be associated with alteration in hypothalamic- pituitary- adrenal (HPA) axis. Taken together, our findings suggest that *IL-1β* gene polymorphisms may play a role in the HPA axis alteration in schizophrenic patients.

Our results showed significant association of rs1143633 with schizophrenia in only females. Although our male sample was not large enough to detect a small relative risk, our data suggest that susceptibility to schizophrenia is more influenced by the *IL-1β* gene variation in females. To our knowledge, no previous studies have examined the gender differences in the association between *IL-1β* gene polymorphisms and schizophrenia. However, gender differences have been reported in the association between schizophrenia and RELA gene [27] encoding the major component of NF-κB, which is activated by IL-1β. Taken together with our results, the influence of IL-1β on susceptibility to schizophrenia may differ between genders. Indeed, gender differences in immunity have been reported in previous studies [36]. IL-1 release from mononucleated cells has been shown to be menstrual phase dependent in females and lower in males [57]. Furthermore, in vitro stimulation of lymphocytes with phytohemagglutinin has shown that females produce more Th2 cytokines than males [58]. Thus, future studies investigating associations of immune-related genes with schizophrenia should take into consideration the possible gender differences.

There are some limitations to this study. The ethnicity of the participants was based on self-reports and was not confirmed by genetic analyses. Our positive results might be derived from sample bias due to population stratification, although the Japanese are a relatively homogeneous population. Furthermore, structured interview such as SCID (Structured Clinical Interview for DSM) was not used for diagnosis in this study. Finally, the function of the *IL-1β* gene SNPs are unclear. Future studies are necessary to elucidate the function and its relationship with the pathogenesis of schizophrenia.

Conclusions

Our results suggest that rs1143633 of *IL-1β* gene is associated with schizophrenia susceptibility in a Japanese population and that the influence of *IL-1β* gene variations on susceptibility to schizophrenia may be greater in females than in males. We obtained no significant evidence for a well-studied polymorphism rs16944 being associated with schizophrenia, which is consistent with previous studies in Asian populations. However, a trend of higher A allele frequency of rs16944 in female patients with schizophrenia leaves open a possibility that a larger study may yield a significant difference. The results of the present study provide further support for the role of IL-1β in the etiology of schizophrenia. Future studies are warranted to replicate the present findings and to reveal the functional role of *IL-1β* gene in pathophysiology of schizophrenia.

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Authors' contributions
DS and HK designed the study and DS wrote the draft of the manuscript. DS, HH, TT, KH, MO, MT, and HK made the diagnosis according to DSM-IV criteria. DS, HH, TT, KH, MO, and HK screened the healthy participants using the Mini International Neuropsychiatric Interview (M.I.N.I.). DS and YI performed the genotyping. HK supervised the data analysis and writing of the paper. TH and NA also supervised the writing of the paper and gave critical comments on the manuscript. All authors contributed to and have approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Phencyclidine-Induced Decrease of Synaptic Connectivity via Inhibition of BDNF Secretion in Cultured Cortical Neurons

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Repeated administration of phencyclidine (PCP), a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor blocker, produces schizophrenia-like behaviors in humans and rodents. Although impairment of synaptic function has been implicated in the effect of PCP, the molecular mechanisms have not yet been elucidated. Considering that brain-derived neurotrophic factor (BDNF) plays an important role in synaptic plasticity, we examined whether exposure to PCP leads to impaired BDNF function in cultured cortical neurons. We found that PCP caused a transient increase in the level of intracellular BDNF within 3 h. Despite the increased intracellular amount of BDNF, activation of Trk receptors and downstream signaling cascades, including MAPK/ERK1/2 and PI3K/Akt pathways, were decreased. The number of synaptic sites and expression of synaptic proteins were decreased 48 h after PCP application without any impact on cell viability. Both electrophysiological and biochemical analyses revealed that PCP diminished glutamatergic neurotransmission. Furthermore, we found that the secretion of BDNF from cortical neurons was suppressed by PCP. We also confirmed that PCP-caused down-regulation of Trk signalings and synaptic proteins were restored by exogenous BDNF application. It is possible that impaired secretion of BDNF and subsequent decreases in Trk signaling are responsible for the loss of synaptic connections caused by PCP.

Keywords: neurotrophin, NMDA receptors, schizophrenia, synaptic function, TrkB signaling pathways

Introduction

Phencyclidine (PCP), a noncompetitive and use-dependent blocker of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, induces psychotic symptoms that are similar to schizophrenia in humans (Allen and Young 1978; Javitt and Zukin 1991). Unlike other psychotomimetic drugs, such as amphetamine, PCP induces negative symptoms (e.g., flattening of affect, alogia, anhedonia, and social withdrawal) and cognitive deficits in addition to positive symptoms (e.g., delusions, hallucinations, and formal thought disorder) of schizophrenia (Andreasen 1995; Olney and Farber 1995; Jentsch and Roth 1999). In rodents, PCP causes schizophrenia-related behaviors, such as disruption in prepulse inhibition (Mansbach and Geyer 1989), stereotyped behavior, and social isolation (Sams-Dodd 1996), increased immobility in forced swimming (Noda et al. 1995), and impaired learning and memory in various maze tasks (Kesner et al. 1983; Handelmann et al. 1987; Danysz et al. 1988; Wass et al. 2006). Extensive reduction in the number of spines in the rat prefrontal cortex has been demonstrated with subchronic PCP treatment (Hajszan et al. 2006), indicating a substantial contribution of abnormal synaptic function to the development of schizophrenia-related behaviors (Mirnics et al. 2001; Frankle et al. 2003; McCullumsmith et al. 2004). Moreover, recent findings suggest that altered expression of genes encoding synapse-associated proteins also play a critical role in the development of schizophrenia (Harrison and Weinberger 2005). Human postmortem studies show reduced dendritic spine density of pyramidal cells in the prefrontal cortex of subjects with schizophrenia (Glantz and Lewis 1997, 2000; Knable et al. 2004). However, the molecular mechanisms underlying the effect of PCP on reduced synaptic connection have not yet been elucidated.

BDNF, a member of the neurotrophin family, plays an important role in synaptic plasticity (Stoop and Poo 1996; Lu 2003; Arancio and Chao 2007, Numakawa et al. 2011) through activation of its receptor Tropomyosin-related kinase B (TrkB) and consequent stimulation of downstream signaling pathways, including mitogen-activated protein/extracellular signal-regulated kinase (MAPK/ERK), phosphoinositide 3-kinase/Akt (PI3K/Akt), and phospholipase C-γ (PLC-γ). We have recently reported important regulatory roles of BDNF in synaptic functions of cortical neurons (Kumamaru et al. 2011). BDNF shows broad expression in the developing and adult mammalian brain (especially, the hippocampus, cerebral cortex, cerebellum, and amygdala) (Ernfors et al. 1990; Hofer et al. 1990; Yan et al. 1997; Conner et al. 1997). As expected, impairment of BDNF/TrkB function has been implicated in the pathogenesis of schizophrenia (Durany and Thome 2004; Angelucci et al. 2005; Lewis et al. 2005), as well as other neuropsychiatric diseases, such as depression (Altar 1999), drug addiction (Davis 2008), Huntington's disease (Zuccato et al. 2001; Gauthier et al. 2004), and Rett syndrome (Chen et al. 2003; Nelson et al. 2008). We found that disrupted-in-schizophrenia 1 (DISC1) and dysbindin, both of which confer susceptibility to schizophrenia, are involved in the regulation of ERK1/2 or Akt signaling (Numakawa et al. 2004; Hashimoto et al. 2006). We have also revealed that glucocorticoid, a stress hormone closely linked to depression (e.g., Kunugi et al. 2006), hampers the synaptic function of BDNF in cortical neurons (Numakawa et al. 2009).

Since expression and secretion of BDNF are facilitated by neuronal activity (Lessmann et al. 2003; Kuczewski et al. 2009),

it is likely that PCP may decrease the expression and/or secretion of BDNF via blockade of neuronal activity. Importantly, increased expression of BDNF was reported in rat hippocampal tissue after acute (Kalinichev et al. 2008) and chronic (Takahashi et al. 2006; Harte et al. 2007) treatment with PCP, although one study reported conflicting results (Semba et al. 2006). In the present study, we investigated changes in expression and secretion of BDNF, activity of downstream signaling cascades stimulated via Trk receptors and synaptic function after PCP exposure.

Materials and Methods

Cortical Cultures and PCP Treatment

Cortical cultures were prepared from postnatal day 1 or 2 old rats (Wistar, SLC, Shizuoka, Japan) as described previously (Numakawa et al. 2002). Dissociated cells were plated at a final density of $5 \times 10^5/cm^2$ on polyethyleneimine-coated culture dishes for immunoprecipitation, immunoblotting, and amino acid measurement. For Ca^{2+} imaging, cortical neurons were cultured on polyethylenimine-coated cover glasses (Matsunami, Osaka, Japan) with FlexiPERM (Greiner Bio-One GmbH, Germany). For immunostaining or electrophysiological recording, neurons were plated on glass-bottom dishes (Matsunami) with a glial feeder layer. The culture medium (5/5 DF) contained 5% fetal bovine serum, 5% heated-inactivated horse serum, 90% of a 1:1 mixture of Dulbecco's modified Eagle's medium, and Ham's F-12 medium. PCP (Sigma-Aldrich, MO) was added to the neurons by bath application at 10–11 days in vitro (DIVs), followed by incubation of the cultures for 3, 6, or 48 h in the presence of PCP before immunocytochemistry, immunoprecipitation, immunoblotting, amino acid measurement and electrophysiological recording. Pure astroglial cultures were prepared as described previously (Hatanaka et al. 1988). Astroglial cells were obtained from cerebral cortex of postnatal day 1 or 2 old rats. All animals were treated according to the institutional guidelines for the care and use of animals.

Immunoblotting

Cells were lysed in sodium dodecyl sulfate (SDS) lysis buffer containing 1% SDS, 20 mM Tris-HCl (pH 7.4), 5 mM ethylene-diamine-tetraacetic acid (EDTA) (pH 8.0), 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide, and 1 mM phenylmethylsulfonyl fluoride. The protein concentration was quantified using a BCA Protein Assay Kit (Pierce Biotechnology Inc., IL), and equivalent amounts of total protein were assayed for each immunoblotting. Primary antibodies were used at the following dilutions: anti-BDNF (1:500, Santa Cruz Biotechnology Inc., CA), anti-TrkB (1:1000, BD Biosciences, NJ), anti-Trk (1:1000, Santa Cruz Biotechnology Inc.), anti-pTyr (1:1000, Upstate, VA), anti-Akt (1:1000, Cell Signaling, MA), anti-pAkt (1:1000, Cell Signaling), anti-ERK (1:1000, Cell Signaling), anti-pERK (1:1000, Cell Signaling), anti-synaptotagmin (1:1000, Calbiochem, Darmstadt, Germany), anti-GluR1 (1:500, Sigma), anti-NR2B (1:500, Sigma), anti-SNAP25 (1:1000, Synaptic Systems, Goettingen, Germany), anti-Bcl-2 (1:1000, BD Biosciences), anti-Bad (1:1000, BD Biosciences), anti-TUJ1 (1:5000, Berkeley Antibody Company, CA), and βactin (1:5000, Sigma) antibodies. The immunoreactivity was quantified by using Lane and Spot Analyzer software (ATTO Corporation, Tokyo, Japan). At least 3 independent series of cultures were used for each set of experiments.

Immunoprecipitation

To detect the phosphorylation of Trk receptors, immunoprecipitation was carried out (Numakawa et al. 2002; Numakawa et al. 2009). After cells were lysed with 1% Triton-X buffer (20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, 1% Triton-X100), anti-Trk antibody (Santa Cruz Biotechnology Inc.) prebound Protein G-Sepharose beads (Amersham Pharmacia, NJ) were mixed with the lysates containing 300 μg of total protein and rotated for 3 h at 4 °C. After 3 washes with the lysis buffer, the proteins that bound the affinity beads were separated by SDS-polyacryl- amide gel electrophoresis (SDS-PAGE) and analyzed for immunoblotting with anti-pTyr (1:1000, Upstate) antibody.

Detection of Cell Surface TrkB

After a gentle wash with ice-cold phosphate-buffered saline (PBS), cells were incubated with 1.1 mg/mL of EZ-Link Sulfo-N-hydroxy- succinimidobiotin (NHS-biotin)-LC-Biotin (Pierce Biotechnology Inc.) in PBS at 4 °C for 20 min. Then, excessive NHS Biotin was quenched with 0.1 M of glycine in PBS and removed by extensive wash with ice-cold PBS. The biotinylated cells were lysed in 1% Triton-X buffer (20 mM of Tris-HCl (pH 7.4), 5 mM of EDTA, 150 mM of NaCl, 1% Triton-X100). Lysates containing equal amounts of protein were mixed with 50 μL of immobilized Neutravidin beads (UltraLink Immobilized NeutrAvidin protein, Pierce Biotechnology Inc.) and incubated at 4 °C for 1 h with gentle rotation. Centrifugated beads were washed 3 times with lysis buffer. The biotin-labeled cell surface proteins were separated by SDS-PAGE and immunoblotted with anti-TrkB antibody (1:1000, BD Biosciences).

Immunocytochemistry

For immunocytochemical staining, neurons were fixed with 4% paraformaldehyde (Sigma) and 4% sucrose in Dulbecco's PBS for 20 min at room temperature. The cells were incubated with PBS containing 0.2% Triton-X (Sigma) for 5 min and blocked by 10% horse serum in PBS for 1 h at 37 °C. Then, anti-MAP2 monoclonal antibody (isotype: IgG1, 1:250, Sigma), anti-BDNF polyclonal antibody (2 μg/μL, produced by Dr Ritsuko Katoh-Semba: Katoh-Semba et al. 1997), anti-GAD67 (glutamic acid decarboxylase 67 kD) monoclonal antibody (1:2000, Millipore, CA) and anti-synaptotagmin monoclonal antibody (isotype IgG2a, 1:100, Chemicon, CA) were applied overnight at 4 °C. BDNF and synaptotagmin were visualized by isotype-specific secondary antibody conjugated with Alexa 488 (1:200, Molecular Probes, CA). MAP2 was visualized by anti-mouse secondary antibody conjugated with Alexa 546 (1:2000, Molecular Probe). A fluorescent microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany) was used to obtain images. When quantification of BDNF immunoreactivity was conducted, we measured mean intensity of randomly selected areas of cell body or primary dendrites by using imaging software Slide Book TM 3.0 (Intelligent Imaging Innovations Inc., CO).

MTT Assay

To calculate cell viability, the metabolic activity of mitochondria was estimated by measuring the mitochondrial-dependent conversion of the tetrazolium salt, MTT (Sigma) (Numakawa et al. 2009). In brief, after treatment of PCP for 48 h, cultured cells were incubated with MTT solution. Two hours later, cultures were lysed and the metabolic activity of the mitochondrial reductase was estimated.

Detection of BDNF Secretion

Following washes with neurobasal medium and 0.1 mg/mL BSA, cultured neurons were incubated with or without PCP (1 μM) in neurobasal medium containing anti-BDNF antibody (2 μg/mL, Santa Cruz Biotechnology Inc.) for 6 h. Then, medium was carefully collected and the secreted BDNF captured by the antibody was immunoprecipitated. After 3 washes with the lysis buffer, BDNF in immunoprecipitates was detected by immunoblotting with the same anti-BDNF antibody (Santa Cruz Biotechnology Inc.) or ELISA assay (BDNF-ELISA E-max; Promega, WI) with another anti-BDNF antibody, a component of the ELISA kit. For detection of BDNF secretion from cortical acute slices, 200-μm-thick coronal sections were prepared using a microtome (VT1000S, Leica, Nussloch, Germany) from prefrontal cortex of postnatal 30–40 days old male rats in ice-cold HEPES-buffered solution (containing 120 mM NaCl, 4 mM KCl, 1.2 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 30 mM glucose, and 20 mM HEPES, pH 7.4). Each slice, which was obtained from right and left cortical hemisphere, was assigned to control and PCP treatment. Freely floating slice sections were incubated with HEPES-buffered solution at 37 °C for 4 h before sampling. Then, after washing several times, secreted BDNF was determined in a similar way used in the culture experiments.

Detection of Amino Acid Neurotransmitters

The amount of amino acid released from cultured neurons was measured as described previously (Numakawa et al. 2002). Briefly, high-performance liquid chromatography (HPLC; Shimazu Co., Kyoto, Japan) was used to measure the amino acids released into the modified HEPES-buffered Krebs Ringer solution (KRH; containing 130 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, 1% bovine serum albumin, and 25 mM HEPES, pH 7.4). After the cultures were washed 3 times with KRH buffer, fresh KRH buffer was added to the cultures and collected without stimulation (1 min) as the basal release. Then, potassium (50 mM KCl for 1 min) was added to the cultures in order to induce depolarization.

Electrophysiology

Whole-cell voltage clamp recordings were performed on cultured cortical neurons using an AxoClamp 2B amplifier (Molecular Devices). Cells were continuously superfused with external solution containing 150 mM NaCl, 4 mM KCl, 2 mM CaCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose, 10 μM glycine (pH = 7.4, 310 mOsm). Miniature excitatory postsynaptic currents (mEPSCs) were isolated by adding picrotoxin (100 μM) and tetrodotoxin (0.5 μM) to the bath. Recordings were performed for 5 min, which enabled us to collect more than 1000 events. Patch electrodes (7–12 MΩ) contained 130 mM Cs-methane sulfonate, 10 mM CsCl, 10 mM $MgCl_2$, 10 mM HEPES, 5 mM MgATP, and 1 mM Na_2GTP (pH 7.3, 300 mOsm). All experiments were carried out at room temperature (27 °C). Cells with an input resistance >200 MΩ (range: 300–800 MΩ) were used. Each neuron was voltage-clamped at −70 mV. For each recording, series resistance and input resistance were continuously monitored, and if these values changed by >15%, the data were discarded. Signals were filtered at 3 kHz and digitized at 10 kHz (Digidata 1320A: Molecular Devices). Off-line analysis of mEPSCs was carried out using Clampfit v 9.2 (Molecular Devices). Miniature events were detected using Mini Analysis software (Synaptosoft) with an amplitude threshold of −5 pA. The events were further inspected visually to exclude inappropriate data, such as overlapped events or events with a noisy baseline.

Imaging of Intracellular Ca^{2+}

Ca^{2+} imaging was performed using fluo-3 dye (Molecular Probes) as previously reported (Numakawa et al. 2002). The changes in the fluo-3 intensity through the fluorescent microscope were analyzed and quantified using Slide Book TM 3.0 (Intelligent Imaging Innovations Inc.) from randomly selected cell bodies. Ca^{2+} imaging experiments were performed at least 3 times for each experimental condition.

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from cultured cells using the RNeasy Plus Kit (QIAGEN, CA) according to the manufacturer's manual. Cells were homogenized by QIAshredder (QIAGEN). To prevent genomic DNA contamination, gDNA Eliminator column (QIAGEN) was used. Total RNA (1 μg) was mixed with SuperScript VILO enzyme and reaction mixes (Invitrogen, CA) in a total volume of 20 μL. After incubation at 25 °C for 10 min, the mixture was heated to 42 °C for 60 min and followed by an inactivation step (85 °C, 5 min). The cDNA solutions were stored at −80 °C until used.

Quantitative PCR

For real-time PCR analyses of mRNA, cDNA was prepared from cultured cortical neurons or pure astrocytes using a TaqMan Cells-to-Ct kit (Applied Biosystems, CA) according to the manufacturer's protocol. Each cDNA was amplified with specific TaqMan Gene Expression Assays (Rn02531967_s1 for rat BDNF; Rn00565046_m1 for rat MAP2; Rn00566603_m1 for rat GFAP: glial fibrillary acidic protein). ABI prism 7000 was used for amplifications. Ct of target mRNA was obtained using Sequence Detection System software (ABI). Serial dilution (1:1, 1:3.3, 1:10, 1:33, and 1:100) of pooled samples was used as a standard. Each gene amplification was normalized with rat GAPDH control (4352338E).

Statistical Analysis

Data are expressed as mean ± standard deviation, and statistical significance was calculated using a one-way ANOVA followed by Scheffe's post hoc test in SPSS ver.18 (SPSS Japan, Tokyo, Japan) if not otherwise specified. The probability values of less than 5% were considered significant.

Results

Transient Increase of Intracellular BDNF Levels by PCP

To determine the acute effects of PCP on neurons, cortical cultures (at DIV 10 and 11) were exposed to PCP, followed by examination of BDNF and its receptor expression. Interestingly, Western blot analyses revealed that 3-h PCP treatment significantly increased levels of intracellular BDNF (14 kD, mature BDNF) (e.g., PCP 1 μM: 152 ± 21.7% of control, $P < 0.01$) (Fig. 1*A,B*). BDNF expression was increased 6 h after 0.1 μM PCP application as well, though statistical significance was not reached (Fig. 1*A,B*). Expression of TrkB and p75 (low affinity receptor for BDNF) were not altered by 3- or 6-h PCP application (Fig. 1*A*, Supplementary Fig. 1*A*). Expression of β-actin and TUJ1 (both are controls) were also intact after PCP exposure (Fig. 1*A*). To check involvement of de novo synthesis in the up regulation of BDNF, we investigated BDNF mRNA after PCP stimulation. Unexpectedly, a significant reduction in BDNF mRNA expression was induced by PCP (1 μM, 3 h) (Supplementary Fig. 1*B*), suggesting that the PCP-induced increase in intracellular BDNF expression is not due to an increase of BDNF translation. Although the cell body size of neurons was not affected (Fig. 1*C*), immunostaining with anti-BDNF antibody showed that the intensity of granular signals in MAP2-positive neurons increased after PCP treatment (1 μM, 3 h) (cell body: 123 ± 10% of control, $P < 0.001$, dendrite: 115 ± 13% of control, $P < 0.001$, *t*-test) (Fig. 1*D,E*). Such granular signals of BDNF were observed in only non-GAD-positive neurons (Supplementary Fig. 1*C*), suggesting that the selected BDNF-positive neurons used to estimate immunoreactivity were glutamatergic neurons. In this study, we confirmed a very small proportion of glial cells in our cortical cultures (Supplementary Fig. 2*A*) and much higher expression of BDNF mRNA in the cortical cultures than that in pure astrocyte cultures. Furthermore, PCP decreased rather than increased the BDNF levels in the pure astrocytes (Supplementary Fig. 2*B*), indicating that the increased expression of BDNF was specific to neurons.

PCP Diminished Activity of BDNF/Trks Signaling

The effect of PCP on activation (phosphorylation) of Trk receptors and downstream signaling pathways was examined. In spite of the increased intracellular BDNF, phosphorylation of Trk receptors was suppressed by 3- and 6-h PCP treatment (PCP 1 μM, 3 h: 49.3 ± 16.3% of control; PCP 1 μM, 6 h: 56.8 ± 10.1% of control, $P < 0.001$ and $P < 0.05$, respectively) (Fig. 2*A*). Three-hour PCP treatment suppressed Trk phosphorylation in all doses tested. Six hours after PCP application, 1–25 μM doses achieved significant inhibition of Trk phosphorylation (Fig. 2*A*). When alterations in cell surface expression of the TrkB receptor were examined, we confirmed that PCP (10 μM) did not change the amount of surface TrkB, while the ligand BDNF indeed reduced surface TrkB levels (Ji et al. 2010) (Fig. 2*B*). As shown in Figure 2*C,D*, activation of Akt (a component of the PI3K pathway) and ERK1/2 (MAPK pathway) were also

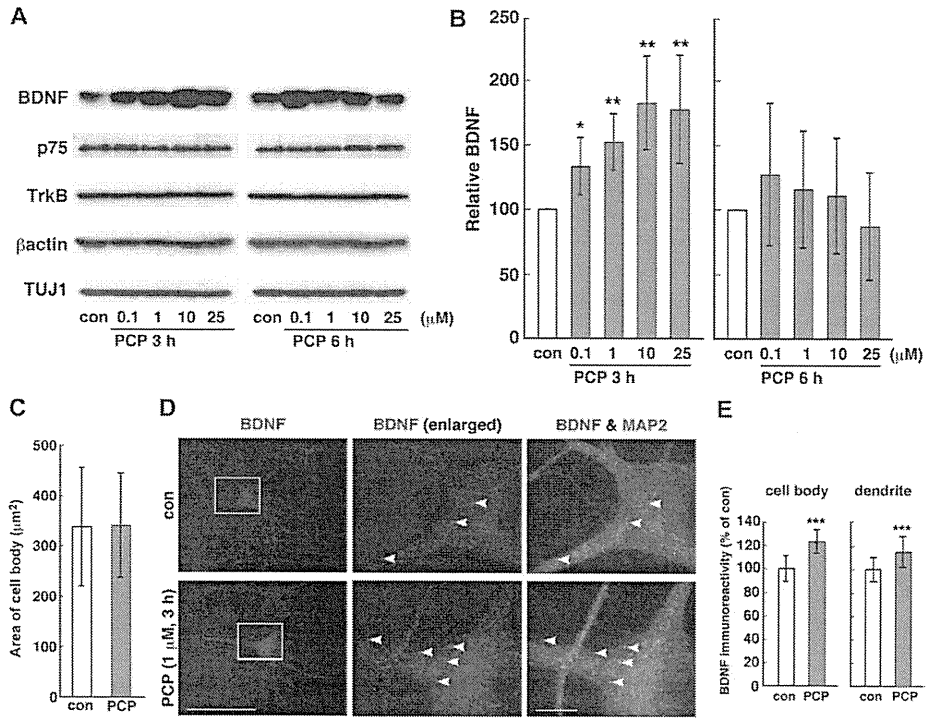

Figure 1. PCP increased levels of intracellular BDNF in cultured cortical neurons. (*A*) BDNF levels (14 kD, mature BDNF) in total lysate were rapidly increased after PCP treatment, whereas expression levels of TrkB and p75 were unchanged. Cultured cortical neurons at DIV 10 and 11 were treated with PCP at indicated doses. Samples were collected 3 or 6 h after PCP addition. TUJ1 and β-actin were represented as controls. (*B*) The mature BDNF expression was quantified. Data represent mean ± standard deviation ($n = 6$, obtained from 6 independent cultures. n indicates the number of experiments if not otherwise specified.). $^{**}P < 0.01$, $^{*}P < 0.05$ versus con (without PCP). Statistical significance was evaluated by one-way ANOVA followed by Scheffe's post hoc test. (*C*) Size of cell body area was not changed by PCP. After 48 h of PCP (10 μM) treatment, neuronal cell body sizes were measured (con: $n = 13$; PCP: $n = 12$, n indicates the number of neurons in each experimental condition). (*D*) Immunocytochemistry revealed a significant increase in BDNF immunoreactivity in cortical neurons in PCP-treated cultures. Cells were immunostained with anti-BDNF and anti-MAP2 antibodies. PCP (1 μM) was applied for 3 h. Left: images after staining with anti-BDNF antibody (green). Bar = 50 μm. Middle: magnified images of insets in Left panels. Right: double staining with anti-BDNF (green) and anti-MAP2 (red) antibodies. Typical BDNF-containing vesicles are indicated by arrowheads. Bar = 10 μm. (*E*) Quantification of BDNF immunoreactivity. Mean intensity of BDNF immunoreactivity in one or two areas of cell body per 1 cell (around 200 μm^2) and in 4–6 primary dendrites (around 50 μm^2) were measured (cell body: con, $n = 20$ and PCP, $n = 22$; primary dendrite: con, $n = 95$ and PCP, $n = 80$; n indicates the number of areas obtained from 14 randomly selected neurons in 4 different dishes of the same culture preparation). $^{***}P < 0.001$ (*t*-test).

decreased by PCP (e.g., PCP 1 μM, 3 h: phosphorylated Akt: 62.9 ± 11.7% of control, $P < 0.001$; phosphorylated ERK1: 61.4 ± 6.7% of control, $P < 0.01$, phosphorylated ERK2: 51.6 ± 10.3% of control, $P < 0.01$), whereas expression of total Akt and ERK1/2 were intact. The reduced activation of Akt and ERK1/2 were maintained until 6 h after PCP treatment (Fig. 2*C,D*). To elucidate which Trk receptor (i.e., TrkA, TrkB, or TrkC) is responsible for the activation of intracellular signaling, cortical neurons were treated with various neurotrophins (Fig. 2*E,F*). BDNF and neurotrophin 4/5 (NT-4/5), both of which are specific ligands for TrkB, strongly activated Trk receptors, Akt and ERK1/2, whereas nerve growth factor (NGF, a specific ligand for TrkA) did not. NT-3 (for TrkC, and weakly for TrkA and TrkB) also demonstrated low activation ability. These data suggest that TrkB signaling is responsible for the activation of Akt and ERK1/2 pathways in our cultures.

Impaired Secretion of BDNF Caused by PCP

In cortical neurons, intracellular BDNF was increased following PCP exposure, whereas activation of Trk and downstream signaling cascades were decreased. To examine whether BDNF secretion was affected by PCP, conditioned medium of cultures incubated in the presence of anti-BDNF antibody was collected. The immunoprecipitated BDNF using conditioned media showed the same molecular weight as that of recombinant mature BDNF in Western blot analysis, and we found reduced amounts of secreted BDNF after PCP exposure (1 μM, 6 h, Fig. 3*A*). A significant reduction in the secreted BDNF was observed (60.7 ± 9.9% of control, $P < 0.001$) in PCP-treated cultures (Fig. 3*B*). Such reduction in immunoprecipitated BDNF was further confirmed by enzyme-linked immunosorbent assay (ELISA) (Fig. 3*C*). Furthermore, we examined BDNF secretion in acute cortical slices and found significant suppression in the amount of secreted BDNF caused by PCP (1 μM, 3 h, Supplementary Fig. 3). It is well known that secretion of BDNF depends on neuronal activity (Hartmann et al. 2001; Lessmann et al. 2003). We tested the effect of glutamate (excitatory neurotransmitter) and tetrodotoxin (Na^+ channels blocker) on BDNF secretion in cultured neurons. As expected, increased BDNF by glutamate and decreased BDNF by TTX in conditioned media were observed (glutamate: 395 ± 114% of control, $P < 0.001$; TTX 49.1 ± 21.0% of control, $P < 0.001$) (Fig. 3*D,E*). These data suggest that PCP repressed the activity-dependent secretion of BDNF from cortical neurons.

Exposure to PCP for 48 h Did Not Affect Neuronal Viability

How does the suppression of BDNF/Trk signaling induced by PCP influence cortical neurons at the cellular and neuronal

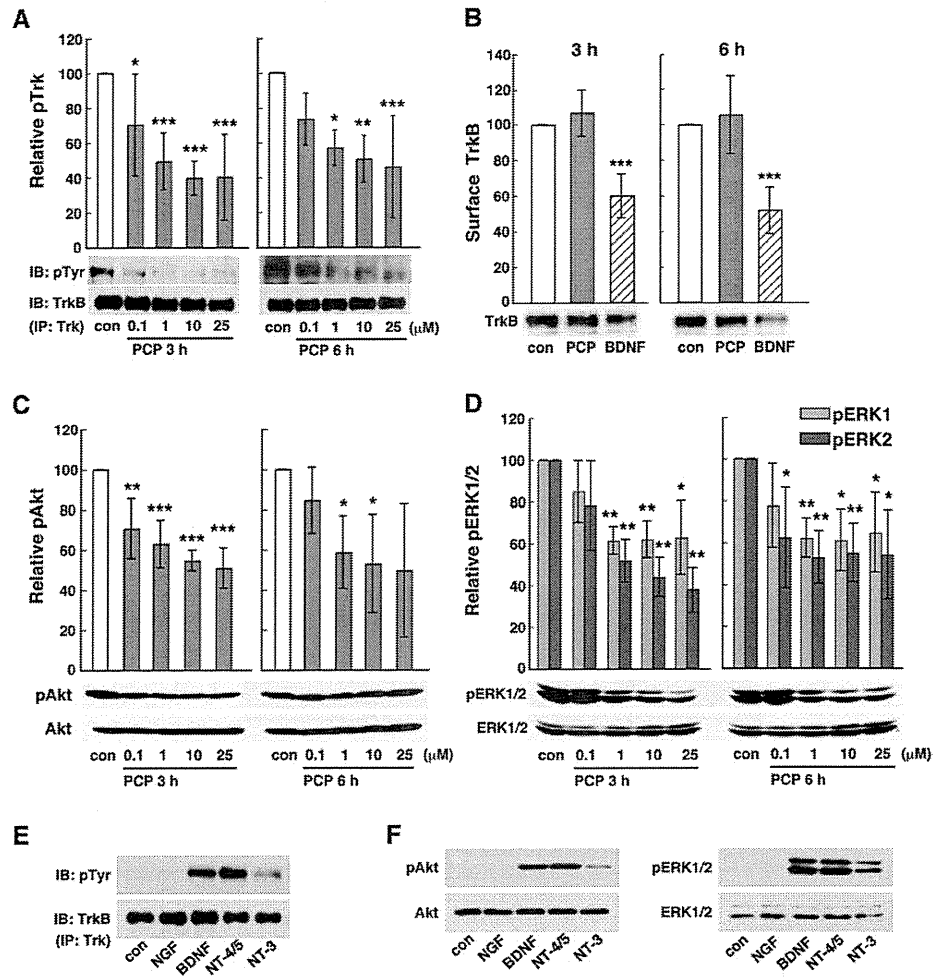

Figure 2. PCP decreased activation of Trk receptors, MAPK/ERK1/2 and PI3K/Akt pathways. (*A*) Phosphorylation of Trk receptors was suppressed after PCP treatment for 3 and 6 h. Cell lysates were immunoprecipitated with anti-Trk antibody. For immunoblotting, anti-phospho-Tyr (pTyr) or anti-TrkB antibodies were used. Phosphorylated Trk receptors (pTrk) were quantified (3 h: $n = 10$; 6 h: $n = 6$, obtained from 5 independent cultures). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus con (one-way ANOVA followed by Scheffe's post hoc test). (*B*) The amount of TrkB receptors on the cell surface after PCP (10 μM) treatment for 3 or 6 h was shown. Note that BDNF (100 ng/mL, positive control for TrkB internalization) exposure decreased surface expression of TrkB while PCP did not. $^{***}P < 0.001$ versus con. (*C,D*) Activation of Akt (a component of the PI3K pathway) and ERK1/2 (MAPK pathway) were reduced up to 6 h after PCP addition. Quantification of phosphorylated Akt (pAkt) (*C*) and ERK1/2 (pERK1/2) (*D*) was conducted ($n = 4$, respectively. Obtained from 4 independent cultures). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus con. (*E,F*) Trk signaling in cortical neurons stimulated by various neurotrophins. (*E*) Following immunoprecipitation with anti-Trk antibody, immunoblotting with anti-pTyr and TrkB antibodies was performed. Each neurotrophin was applied at 100 ng/mL. Note that BDNF and NT-4/5 significantly stimulated Trk receptors in cortical neurons. (*F*) PI3K/Akt (Left) and MAPK/ERK1/2 (Right) pathways.

network level? To approach this issue, the survival of neurons after exposure to PCP was examined. After 48 h of exposure to 1 μM PCP, cortical neurons were immunostained with anti-MAP2 antibody (Fig. 4*A*). Quantified data of MAP2-positive living cells revealed no difference in neuronal survival between control and PCP-treated cultures (Fig. 4*B*). MTT assay also indicated that PCP (0.1–25 μM, 48 h) treatment did not affect cell viability (Fig. 4*C*). As shown in Fig. 4*D*, expression of Bcl-2, an antiapoptotic protein, and Bad, a proapoptotic protein, were unchanged by PCP (1 μM, 48 h).

PCP Diminished the Number of Presynaptic Sites and Synaptic Transmission

We next investigated the effect of PCP (48 h) on synaptic function. Western blot analysis revealed decreased expression of presynaptic proteins (synaptotagmin and synaptosome-associated protein of 25 kD [SNAP25]) and postsynaptic glutamate receptors (NR2B and GluR1) (Fig. 5*A*). Immunostaining with anti-synaptotagmin antibody also demonstrated a decrease in the number of presynaptic puncta after PCP (1 μM, 48 h) exposure (13.8 ± 2.9 per 50 μm of dendrite in control and 8.3 ± 3.0 in PCP, $P < 0.001$) (Fig. 5*B,C*). Importantly, basal and depolarization-induced release of glutamate were both decreased after 48 h of PCP treatment (Fig. 5*D*), suggesting that PCP decreases the number of glutamatergic synapses. Electrophysiological recordings on cultured cortical neurons revealed that PCP (1 μM, 48 h) reduced the frequency of mEPSCs in both NMDA receptor and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor components (Fig. 6*A,B*). Cumulative plots also indicated a shift to longer intervals in both AMPA receptor- and NMDA receptor-mediated mEPSCs after PCP treatment (Fig. 6*C*), while the amplitude of mEPSCs was not changed (Fig. 6*D*).

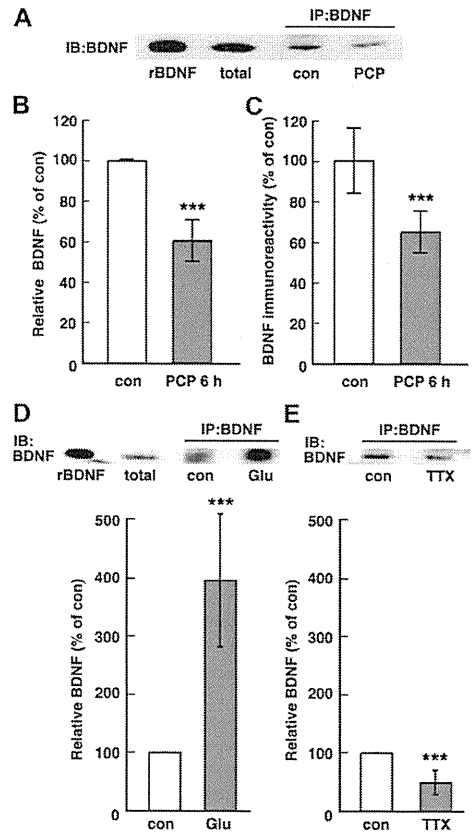

Figure 3. PCP decreased secretion of BDNF from cortical neurons. Secreted BDNF into culture medium was immunoprecipitated with anti-BDNF antibody. In the presence of anti-BDNF antibody (2 μg/mL), cortical neurons were incubated with or without PCP at 1 μM for 6 h, followed by immunoprecipitation. A representative Western blot (*A*) and quantified data (*B*) were shown (n = 12, from 5 independent cultures). ***$P < 0.001$ versus con (t-test). rBDNF: recombinant BDNF; total: total lysates (10% input). (*C*) ELISA analysis also showed a decreased amount of BDNF secretion by PCP (con: n = 11; PCP: n = 12, n indicates the number of dishes for each experimental condition). (*D*, *E*) Glutamate increased secretion of BDNF while TTX decreased it. Cortical neurons were treated with glutamate (1 μM, 15 min) (*D*) or TTX (3 μM, 6 h) (*E*). (Glutamate: n = 6, TTX: n = 5, from 5 independent cultures, respectively). ***$P < 0.001$ versus con (t-test).

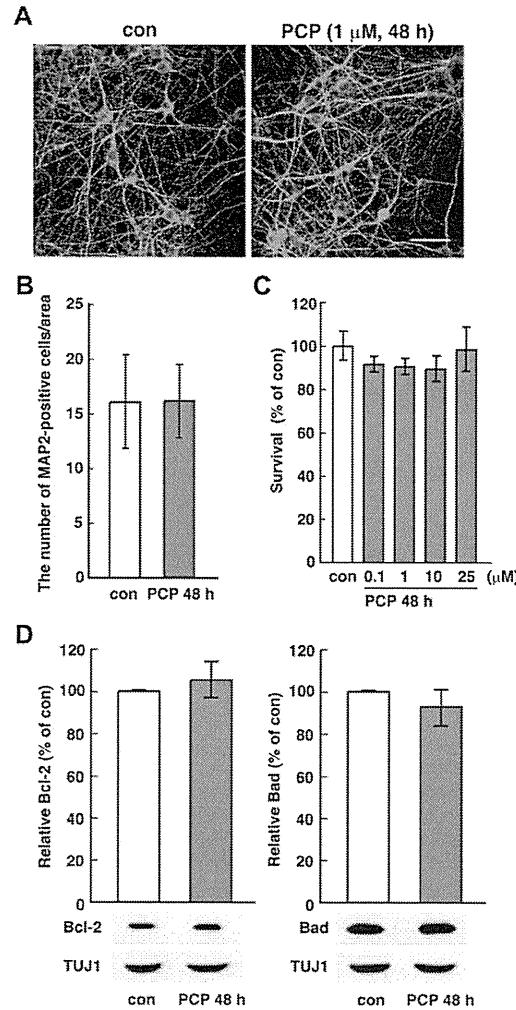

Figure 4. PCP did not affect neuronal viability. (*A*) Forty-eight hours after PCP (1 μM) treatment, cortical neurons were stained with anti-MAP2 antibody. Bar = 50 μm. (*B*) These MAP2-positive cells were counted (n = 18, n indicates the number of randomly selected 250 × 250 μm^2 fields from 5 dishes for each experimental condition). (*C*) PCP (0.1–25 μM, 48 h) treatment did not affect the cell viability, which was determined by MTT assay (n = 4). Normalization to control was carried out. (*D*) Both Bcl-2 (an antiapoptotic protein) and Bad (a proapoptotic protein) levels were not altered by PCP (1 μM, 48 h) (n = 4). TUJ1 is represented as control.

Exogenous BDNF Prevented PCP's Suppression of Intracellular Signaling and Synaptic Protein Expression

We investigated whether coapplication of BDNF with PCP could prevent the reduction in ERK1/2 and Akt activation at 3 h later as well as prevent the decrease in synaptic protein expression at 48 h later. As expected, exogenous BDNF (10 ng/mL) completely prevented the reduction of intracellular signaling (ERK 1/2 and Akt activation, Fig. 7*A*) and of synaptic proteins (synaptotagmin, SNAP25, NR2B, and GluR1, Fig. 7*B*). Delayed BDNF application at 3 h after PCP addition also prevented down regulation of synaptic proteins caused by PCP (Supplementary Fig. 4). These data suggest that the PCP-induced loss of synaptic connectivity is due to the impaired secretion of endogenous BDNF.

PCP Blocked Intracellular Ca^{2+} Mobilization and Other NMDA Receptor Antagonists Induced Intracellular BDNF Increase

Increase in the intracellular Ca^{2+} concentration is required for the activity-dependent secretion of BDNF (Hartmann et al. 2001; Lessmann et al. 2003). Thus, we assessed the possibility that the PCP-suppressed BDNF secretion is due to an inhibition of Ca^{2+} influx mediated by glutamate receptors. First, as shown in Fig. 8*A*, change in levels of glutamate receptors including NR2A, NR2B, and GluR1 during in vitro maturation were determined. We found that these proteins gradually increased during in vitro maturation, while very low expression levels at DIV 4 and 5 occurred (Fig. 8*A*). Synaptotagmin and SNAP25, presynaptic proteins, also increased during in vitro maturation in our cultures. Previously, we reported that cultured cortical neurons develop spontaneous Ca^{2+} oscillations during in vitro maturation, and the endogenous phenomenon is mediated via glutamatergic neurotransmission (Numakawa et al. 2002). Therefore, we determined whether PCP affected spontaneous Ca^{2+} oscillations at DIV 12 and found that PCP treatment (1 μM, 3 h) dramatically reduced the endogenous Ca^{2+} activity (Fig. 8*B,C*). In some cases, we observed a resting series of sister

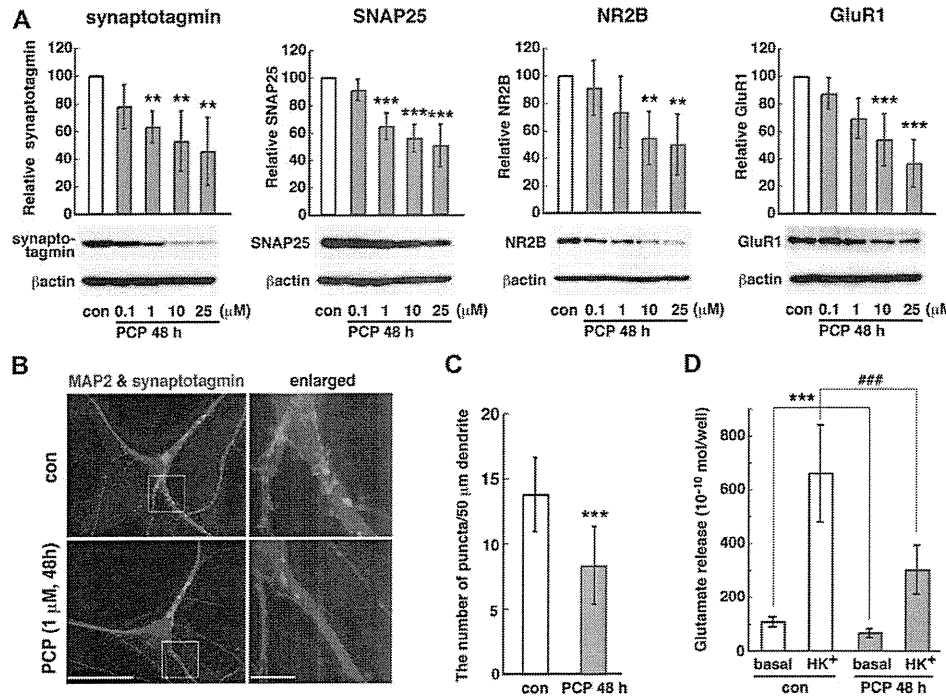

Figure 5. PCP reduced the number of synaptic sites, expression of synaptic proteins, and release of glutamate. (*A*) The amount of synaptic proteins, including synaptotagmin, SNAP25, NR2B, and GluR1, were reduced by PCP. Samples were collected and analyzed after 48 h PCP (0.1–25 μM) treatment. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ versus con (one-way ANOVA followed by Scheffe's post hoc test). ($n = 7$, from 5 independent cultures.) (*B*) The number of presynaptic sites was decreased by PCP (1 μM, 48 h). Representative images after synaptotagmin (red) and MAP2 (green) immunostaining are shown. Left: Bar = 50 μm, Right: inset in Left panels was magnified. Bar = 10 μm. (*C*) Synaptotagmin-positive presynaptic sites were counted. Thirty-eight randomly selected primary dendrites (per 50 μm) from 14 neurons in 4 randomly selected dishes for each experimental condition were selected. $***P < 0.001$ versus con (*t*-test). (*D*) Glutamate release from cultured neurons was diminished by exposure to PCP. Glutamate from cortical cultures was collected for 1 min as nonstimulated basal and as high potassium-stimulated release. HK^+; high potassium (KCl, 50 mM) condition. $***P < 0.001$, $^{###}P < 0.001$ (*t*-test). $n = 6$. *n* indicates the number of dishes for each experimental condition.

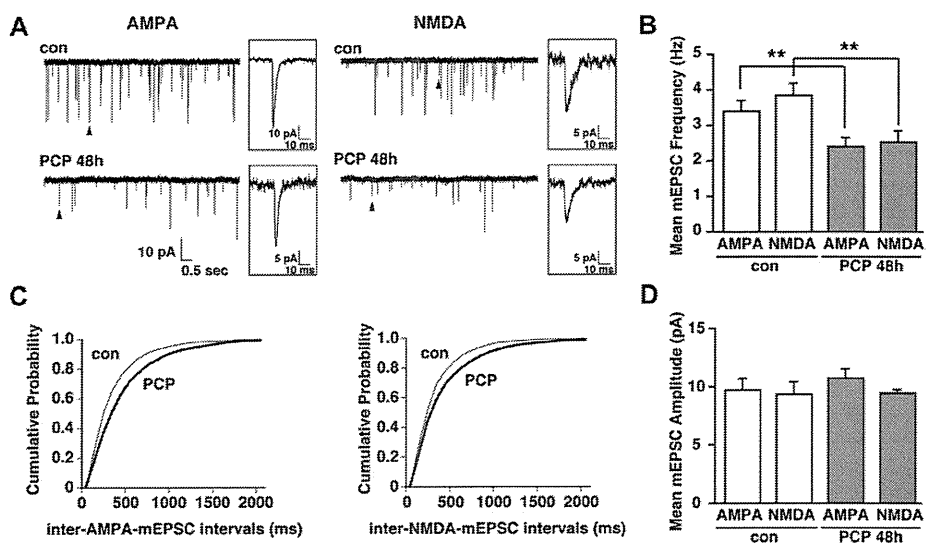

Figure 6. PCP decreased the frequency (but not amplitude) of mEPSCs. Cortical cultures were exposed to PCP (1 μM, 48 h), followed by recording of mEPSCs. (*A*) AMPA component of mEPSCs (AMPA-mEPSC) was recorded in control- and PCP-treated neurons in the presence of D-APV (25 μM) (Left). NMDA component of mEPSPs (NMDA-mEPSC) in both control and PCP conditions in the presence of CNQX (5 μM) with Mg^{2+} free was also measured (Right). Typical mEPSC for each condition (arrowhead) are shown in the inset. (*B*) Mean mEPSC frequency for control cells and PCP-treated cells. Error bars represent SEM. $**P < 0.05$ (*t*-test). (*C*) Cumulative probability histogram for inter-AMPA-mEPSC (Left) and inter-NMDA-mEPSC intervals (Right) from control (thin) and PCP treatments (solid). Each data point represents the mean from 5 cells. (*D*) Mean amplitude of mEPSC in control and in PCP treatments. *n* indicates the number of cells obtained from 6 to 10 independent cultures.

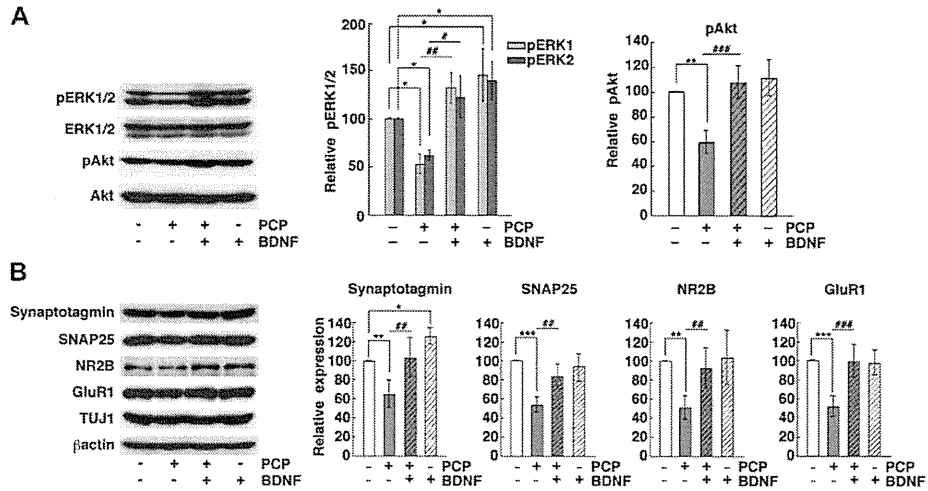

Figure 7. Exogenous BDNF prevented down regulation of intracellular signaling and synaptic protein expression. BDNF (10 ng/mL) was added immediately after PCP (10 μM) application. (*A*) Exogenous BDNF prevented the negative effect of PCP on ERK 1/2 and Akt activation. Samples were collected at 3 h after PCP application. (n = 4, from 2 independent cultures.) $^{**}P < 0.01$, $^{*}P < 0.05$ versus con; $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus PCP (one-way ANOVA followed by Scheffe's post hoc test). (*B*) Decrease in levels of synaptic proteins, including synaptotagmin, SNAP25, NR2B, and GluR1 after PCP treatment (10 μM, 48 h) did not occur in the presence of BDNF (10 ng/mL). TUJ1 and β-actin are controls. (n = 5, from 3 independent cultures.) $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus con; $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$ versus PCP (one-way ANOVA followed by Scheffe's post hoc test).

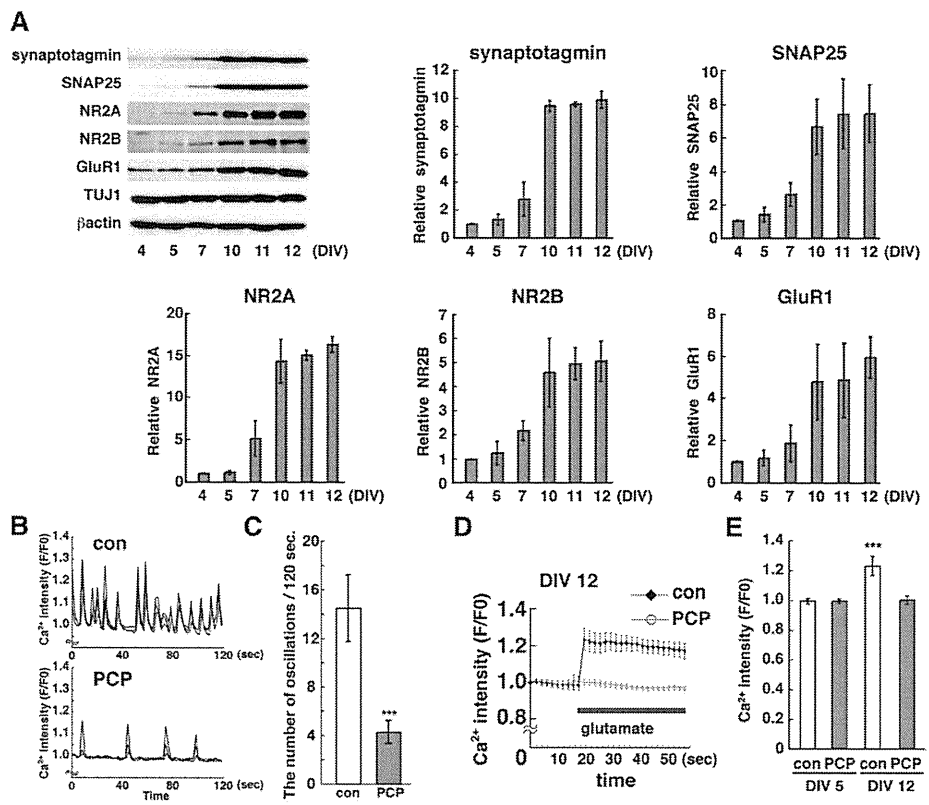

Figure 8. PCP inhibited intracellular Ca^{2+} elevation in mature cortical neurons. (*A*) Developmental change in the expression of glutamate receptors (NR2A, NR2B, and GluR1), in addition to synaptotagmin and SNAP25, were shown. Expression of these synaptic proteins was gradually increased during in vitro maturation and reached maximum levels around DIV 10. (n = 3, from 3 independent cultures). TUJ1 and β-actin are controls. (*B*) Spontaneous Ca^{2+} oscillations were decreased in cultured cortical neurons in the presence of PCP (1 μM, 3 h). Traces were obtained from 3 neurons at DIV 12 in the same dish for each condition. The fluorescence ratio (F/F_0, F_0; basal intensity) was calculated. (*C*) The number of the frequency in Ca^{2+} oscillations was counted. (n = 6, n indicates the number of dishes of a sister culture). $^{***}P < 0.001$ versus con. (*D*) Glutamate-triggered intracellular Ca^{2+} elevation was blocked by PCP. PCP (1 μM) was applied 3 h before glutamate (1 μM) stimulation. DIV 12. (*E*) Quantified data of the evoked Ca^{2+} in the presence or the absence of PCP at DIV 5 or at DIV 12. Summarized data from 50 randomly selected cells for each experimental condition. $^{***}P < 0.001$ versus con. (one-way ANOVA followed by Scheffe's post hoc test).