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# Failure to replicate the association between *NRG1* and schizophrenia using Japanese large sample

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**Abstract**

Systematic linkage disequilibrium (LD) mapping of 8p12–21 in the Icelandic population identified neuregulin 1 (*NRG1*) as a prime candidate gene for schizophrenia. However, results of replication studies have been inconsistent, and no large sample analyses have been reported. Therefore, we designed this study with the aim of assessing this putative association between schizophrenia and *NRG1* (especially $HAP_{ICE}$ region and exon region) using a gene-based association approach in the Japanese population.

This study was a two-stage association analysis with a different panel of samples, in which the significant association found in the first-set screening samples (1126 cases and 1022 controls) was further assessed in the confirmation samples (1262 cases and 1172 controls, and 166 trio samples). In the first-set scan, 60 SNPs (49 tagging SNPs from HapMap database, four SNPs from other papers, and seven SNPs detected in the mutation scan) were examined.

One haplotype showed a significant association in the first-set screening samples (Global *P*-value = 0.0244, uncorrected). However, we could not replicate this association in the following independent confirmation samples. Moreover, we could not find sufficient evidence for association of the haplotype identified as being significant in the first-set samples by imputing ungenotyped SNPs from HapMap database.

*Abbreviations: NRG1*, neuregulin 1; SNP, single nucleotide polymorphism; GGF2, glial growth factor 2; LD, linkage disequilibrium; dHPLC, denaturing high performance liquid chromatography; MAF, minor allele frequency; TDT, transmission disequilibrium test; UTR, untranslated region.

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These results indicate that the positionally and functionally attractive regions of *NRG1* are unlikely to contribute to susceptibility to schizophrenia in the Japanese population. Moreover, the nature of our results support that two-stage analysis with large sample size is appropriate to examine the susceptibility genes for common diseases.


*Keywords:* Schizophrenia; Neuregulin 1; Association study; False positive; Linkage disequilibrium

## 1. Introduction

Schizophrenia is a common psychiatric disorder with a lifetime prevalence of 1% worldwide. Family, twin and adoption studies show conclusive evidence of a substantial genetic component in this disorder. Progress towards detecting these genetic elements is now being made (Harrison and Weinberger, 2005).

The neuregulin 1 gene (*NRG1*) was first reported to be a prime candidate gene for schizophrenia in the Icelandic population (Stefansson et al., 2002). The significant association of a haplotype was detected in the 5′-region of glial growth factor 2 (GGF2) isoforms, and this at-risk haplotype, consisting of five single nucleotide polymorphisms (SNPs) and two microsatellites, was named as $HAP_{ICE}$. Several subsequent studies provided the following evidence to support this association with schizophrenia.

Firstly, the location of this gene corresponds to the linkage regions for schizophrenia (8p12–21, OMIM: SCZ5), which were identified by recent meta-analyses of genome-wide linkage studies (Badner and Gershon 2002; Lewis et al., 2003). Secondly, recent evidence suggests that mutation within the *NRG1* region might give rise to functional alterations that are in line with the neurodevelopmental hypothesis and glutamate/GABA hypothesis of schizophrenia (Corfas et al., 2004).

Thirdly, several independent association studies have replicated the original significant association found by Stefansson et al. (2002). However, the results of replication studies using the identical number or fewer sets of markers have been inconsistent. Thus, while some research groups did not report any association (Iwata et al., 2004), other studies showed a positive association but showed different 'at-risk' haplotypes to be associated with schizophrenia (Harrison and Law, 2006).

These inconsistent results could stem from the possibility that *NRG1* is not involved in the etiology of schizophrenia in all populations. However, this inconsistency could be a consequence of the unique structure of the human genome. In other words, differences in linkage disequilibrium (LD) among populations may also be responsible for the differences in the results, and the negative findings may only indicate a failure to reflect the actual predisposing variants due to the differences in populations.

Therefore, gene-wide (or region-wide) replication analysis based on LD pattern within the *NRG1* region is essential to detect an association in a certain population setting (Neale and Sham, 2004). In such analyses, particular attention should be paid to selection of genetic variants which adequately reflect the LD background in the targeted population (e.g. tagging SNPs).

Although the above-mentioned LD-based association analysis is based on the common disease–common variant hypothesis, one study reported an association between *NRG1* and schizophrenia from the standpoint of the common disease–rare variant hypothesis (Walss-Bass et al., 2006). The authors scanned the whole exon region, detected a non-synonymous SNP in exon 11, and showed a significant association of this SNP with schizophrenia. Detection of rare but potent functional variants relies on large mutation scan samples; however, such rare variants may also differ among populations (Pritchard, 2001).

Thus, in this study, we first focused on two attractive regions: the 5′ regions of GGF2, where the original study showed the association (henceforth referred to as '$HAP_{ICE}$ region') and the exon region (henceforth referred to as 'exon region'). In the exon region, prior to association analysis of tagging SNPs, we performed a mutation scan in order to detect the existence of possible potent functional variants in the ethnic samples. In addition, this study was a two-stage association analysis with a different panel of samples, in which the significant association in the first-set screening samples (1126 cases and 1022 controls) was further assessed in confirmation samples (1262 cases, 1172 controls, and 166 trio samples). This approach was adopted in order to avoid the possibility of type I or type II error.

## 2. Methods and materials

### 2.1. Subjects

Two independent sample sets were used in this study. For the first-set screening analysis, 1126 patients with schizophrenia (627 male and 499 female; mean age ± standard deviation (SD) 47.0 ± 15.3 years) and 1022

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healthy controls (530 male and 492 female; 38.8± 14.5 years) were examined. Confirmation analysis was conducted with three samples consisting of: (a) 1262 patients with schizophrenia (662 male and 600 female; 49.1±14.5 years) (b) 1172 controls (576 male and 596 female; 41.7±14.3 years), and (c) 166 family trios samples (of the patients, 91 male and 75 female; 30.0± 8.3 years).

The subjects for mutation search were 96 patients with schizophrenia. These subjects were also included in the first-set samples. 385 cases and 336 controls in the first-set samples, and 349 cases (including 84 cases from family samples) and 424 controls in confirmation samples are identical to those in our previous report (Iwata et al., 2004) and Fukui et al.'s (2006) report, respectively.

Characterization details and psychiatric assessment of these subjects were as follows. The patients were diagnosed according to DSM-IV criteria consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews. All subjects were ethnically Japanese.

After the study had been described to subjects, written informed consent was requested from each. This study was approved by the ethics committees at Fujita Health University, Teikyo University, Okayama University, Osaka University, Niigata University and Nagoya University Graduate School of Medicine.

### 2.2. Mutation scan

We performed denaturing high performance liquid chromatography (dHPLC) analysis, details of which can be seen in a previous paper (Ikeda et al., 2005). Primer sequences were designed in accordance with another report (Walss-Bass et al., 2006).

### 2.3. Tagging SNP selection

We included the three signal SNPs (SNP8NRG221533, SNP8NRG241930 and SNP8NRG243177) from the report of Stefansson et al. (2002) (we excluded SNP8NRG221132 and SNP8NRG433E1006 from the first-set analysis due to low minor allele frequencies (MAFs) in the Japanese population), one positive SNP from the report of Walss-Bass et al. (2006), and SNPs we detected in the mutation scan. Next we consulted the HapMap database (release#19, population: Japanese in Tokyo (JPT), MAF: more than 0.05). In this step, we determined the boundaries of the '$HAP_{ICE}$ regions' that cover 5′ regions including 19,425 bp and 155,564 bp downstream (3′) from the significant SNPs (SNP8NRG221132 and SNP8NRG433E1006, respectively) in Stefansson's report (Table 1 and Supplementary Fig. 1) (Stefansson et al., 2002), and of the 'exon regions' that cover 5′ regions including 120,576 bp from the first exon and 3510 bp downstream 3′ from the last exon (GenBank accession No. NT_007995: Table 2 and Supplementary Fig. 2). Then fifteen and thirty-four 'tagging SNPs' for the $HAP_{ICE}$ regions and exon regions, respectively were selected with the criterion of an $r^2$ threshold greater than 0.8 in 'Aggressive tagging: use 2- and 3-markers haplotypes' mode of the 'Tagger' program (de Bakker et al., 2005), a function of HAPLOVIEW software (Barrett et al., 2005).

### 2.4. SNP genotyping

All SNPs were genotyped by TaqMan assay (Applied Biosystems Japan Ltd, Tokyo).

The genotyping of C#5, C#6, C#7 (which were positive SNPs in the first-set screening analysis) was done with 768 randomly selected samples (384 cases and 384 control subjects) with direct sequencing to check for genotyping error. Detailed information including primer sequences of custom TaqMan SNP genotyping assays can be seen in Supplementary Tables 1 and 2.

Table 1
First-set case control analysis of $HAP_{ICE}$ region

| Markers | SNP ID | *P*-values | | |
|---|---|---|---|---|
| | | 1-window | 2-windows | 3-windows |
| $HAP_{ICE}$#1 | rs12674974 | .0794 | | |
| $HAP_{ICE}$#2 | rs4513929 | .846 | .181 | |
| $HAP_{ICE}$#3 | SNP8NRG221533 | .188 | .384 | .196 |
| $HAP_{ICE}$#4 | rs10096573 | .200 | .397 | .620 |
| $HAP_{ICE}$#5 | rs4733263 | .310 | .414 | .462 |
| $HAP_{ICE}$#6 | rs4733263 | .274 | .616 | .267 |
| $HAP_{ICE}$#7 | SNP8NRG241930 | .724 | .399 | .578 |
| $HAP_{ICE}$#8 | SNP8NRG243177 | .288 | .113 | .326 |
| $HAP_{ICE}$#9 | rs4733267 | .769 | .520 | .492 |
| $HAP_{ICE}$#10 | rs13277456 | .862 | .889 | .190 |
| $HAP_{ICE}$#11 | rs13274954 | .457 | .736 | .847 |
| $HAP_{ICE}$#12 | rs12677942 | .312 | .670 | .255 |
| $HAP_{ICE}$#13 | rs4403369 | .0803 | .271 | .128 |
| $HAP_{ICE}$#14 | rs4566990 | .625 | .268 | .548 |
| $HAP_{ICE}$#15 | rs13270788 | .541 | .628 | .525 |
| $HAP_{ICE}$#16 | rs1503491 | .813 | .730 | .699 |
| $HAP_{ICE}$#17 | rs2202262 | .704 | .866 | .0960 |
| $HAP_{ICE}$#18 | rs10087212 | .682 | .324 | .0653 |
| $HAP_{ICE}$#4–#5 | | .414 | | |
| $HAP_{ICE}$#14–#16 | | .247 | | |
| $HAP_{ICE}$#15–#16 | | .730 | | |

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Table 2
First-set case control analysis of exon region

| Markers | SNP ID | *P*-values[a] | | |
|---|---|---|---|---|
| | | 1-window | 2-windows | 3-windows |
| C#1 | rs10503915 | .116 | | |
| C#2 | rs7016691 | .231 | .0603 | |
| C#3 | rs11782671 | .472 | .371 | .349 |
| C#4 | rs10103930 | .168 | .474 | .296 |
| C#5 | rs10503917 | .699 | .322 | .508 |
| C#6 | rs10107065 | .765 | .628 | .0935 |
| C#7 | rs6468118 | .138 | .138 | **.0244** |
| C#8 | rs7000590 | .0939 | .154 | .174 |
| MS1 | rs7820838 | .110 | .107 | .158 |
| MS2 | rs7834206 | .149 | .142 | .181 |
| C#9 | rs4236709 | .0786 | .0879 | .145 |
| C#10 | rs13260545 | .0994 | .187 | .352 |
| C#11 | rs4316112 | .948 | .248 | .403 |
| C#12 | rs2439305 | .196 | .144 | .0984 |
| C#13 | rs7826814 | .715 | .130 | .132 |
| C#14 | rs2466064 | .690 | .851 | .129 |
| MS3 | rs3924999 | .162 | .313 | .436 |
| C#15 | rs10954864 | .803 | .113 | .0699 |
| C#16 | rs2439281 | .965 | .969 | .602 |
| C#17 | rs9642729 | .0680 | .0725 | .301 |
| C#18 | rs12547858 | .0801 | .0988 | .137 |
| C#19 | rs10098373 | .801 | .457 | .523 |
| C#20 | rs10095694 | .380 | .835 | .654 |
| MS4 | rs3735774 | .762 | .727 | .872 |
| C#21 | rs2466058 | .372 | .727 | .718 |
| C#22 | rs2466052 | .379 | .526 | .587 |
| C#23 | rs2466046 | .187 | .286 | .509 |
| C#24 | rs10503923 | .546 | .372 | .431 |
| C#25 | rs2466084 | .310 | .473 | .203 |
| C#26 | rs2976515 | .253 | .551 | .197 |
| C#27 | rs4445183 | .702 | .654 | .563 |
| C#28 | rs2919377 | .151 | .484 | .500 |
| C#29 | rs2919375 | .819 | .341 | .455 |
| MS5 | rs3735776 | .740 | .222 | .182 |
| C#30 | rs7007436 | .711 | .758 | .129 |
| C#31 | rs3757934 | .758 | .815 | .866 |
| MS7 | rs4733376 | .379 | .421 | .562 |
| C#32 | rs4360253 | .357 | .336 | .357 |
| C#33 | rs7005288 | .864 | .893 | .789 |
| C#34 | rs6992642 | .569 | .812 | .738 |
| MS6 (C#24–#30)[b] | rs17731664 | .772 | | |
| C#5–#11–#14 | | 1.00 | | |
| C#5–#14 | | .180 | | |
| C#16–#27 | | .751 | | |
| C#23–#26–#28 | | .245 | | |

[a] Bold number represents significant *P*-value.
[b] MS6 could be represented by the haplotypes constructed by C#24–30.

### 2.5. Statistical methods for conventional association analysis

In the case–control samples, the marker–trait association was evaluated with the $\chi^2$ test in allele- and genotype-wise analyses. Haplotype frequencies were estimated in a 2- to 3-marker sliding window fashion by EM algorithm and Log likelihood ratio tests were performed for Global *P*-values with COCAPHASE program version 3.06 (Dudbridge, 2003). In the family samples, the transmission disequilibrium test (TDT) and 3-marker haplotype analyses were performed with the TDTPHASE program version 3.06 (Dudbridge, 2003). In these haplotype-wise analyses, rare haplotypes (less than 0.05) of cases and controls were excluded from the association analysis in order to provide greater sensitivity and accuracy.

The significance level was set at $P<0.05$.

### 2.6. Imputation of ungenotyped SNPs

Our conventional haplotype-wise analysis was done in a sliding window fashion, since our selection for tagging SNPs was not based on the haplotype block concept. Although this type of haplotype-wise analysis does not adapt to the degree of LD, so that it is unclear which markers should be considered jointly, it results in a higher level of statistical power since it can reflect unknown SNPs that were not included in the analysis. Considering this, we included a recently developed method, imputation, to test for any SNPs that reflect the significant haplotypes (Marchini et al., 2007). The IMPUTE program imputes the genotypic distribution of un-observed SNPs using observed SNP information (60 SNPs used in the screening scan) and the HapMap database (fine-scale recombination map, haplotype for JPT/CHP).

The targeted region for imputation was limited to within known recombination hot spots, because our data targeted only the $HAP_{ICE}$ and exon regions.

After imputation, we applied a Bayesian test with an additive model to assess the association using SNPTEST software (Marchini et al., 2007). Default values were used in all settings needed in IMPUTE and SNPTEST (e.g. effective population size for JPT/CHP, buffer, call threshold for calling genotyped SNPs and number of samples of genotypes that should be used for Bayesian tests).

Table 3
Individual haplotype analyses from significant Global *P*-values in first-set samples

| | haplotypes | Case Freq (%) | Con Freq (%) | *P*-value | Global *P*-value |
|---|---|---|---|---|---|
| C#5–6–7 | 1–1–1 | 9.36 | 11.8 | .0104 | .0244 |
| | 1–1–2 | 15.6 | 13.6 | .0896 | |
| | 1–2–2 | 65.8 | 65.5 | .886 | |
| | 2–1–1 | 7.21 | 6.27 | .300 | |

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### 2.7. Power calculation

Power calculation was performed with a web-based statistical program, Genetic Power Calculator (Purcell et al., 2003). Power was estimated under a multiplicative model of inheritance, assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values observed in control samples.

## 3. Results

### 3.1. Mutation scan and first-set association analysis

We detected seven SNPs through dHPLC analysis of the exon region (MS1–7: Table 2). One of them, MS3 (rs3924999), is a non-synonymous SNP (Gly38Arg) and had shown a significant association in the Chinese population (Yang et al., 2003). The other SNPs were located in an untranslated region (UTR) or branch site, and may therefore have a functional effect (Table 2).

Next, 49 SNPs and 7 haplotypes were selected as Tagging SNPs from the HapMap database. These SNPs are located in the $HAP_{ICE}$- and coding regions based on the HapMap database (Tables 1 and 2).

Consequently, by involving 11 SNPs (the 7 SNPs we detected and 4 SNPs reported in other papers (Stefansson et al., 2002; Walss-Bass et al., 2006)), a total of 60 SNPs were genotyped in the first-set screening samples (however, since we were unable to design a genotyping method for one SNP that we detected (MS6) by TaqMan Assay by Design (Applied Biosystems), we determined the genotype distribution of some samples (192 cases and 192 controls) using a direct sequencing method. With these samples we confirmed that MS6 could be represented by the haplotypes constructed by C#24–30 in LD evaluation.).

The SNP for which significance was shown in the report of Walss-Bass et al. (2006) was not polymorphic in our samples.

Allele- and genotype-wise analyses did not show association either the $HAP_{ICE}$ region or the exon region. In this haplotype-wise analysis, 3-marker haplotypes of C#5–6–7 were associated with schizophrenia (Global $P$-value=0.0244, uncorrected: Tables 1, 2 and 3, Supplementary Tables 3 and 4). The genotyping of C#5, C#6, C#7 in a subset of the screening samples was re-confirmed by direct sequencing, and the results were perfectly identical to those shown by TaqMan assay. Hence, we speculate that it was unlikely that genotyping error had occurred.

### 3.2. Imputation of ungenotyped SNP for first-set samples

Data for ungenotyped SNPs could not provide sufficient evidence for association in either region (Fig. 1). In particular, the weights of evidence for the regions near the significant haplotypes in first-set samples were less than one. Since weights of evidence of at least four are required for evidence for association

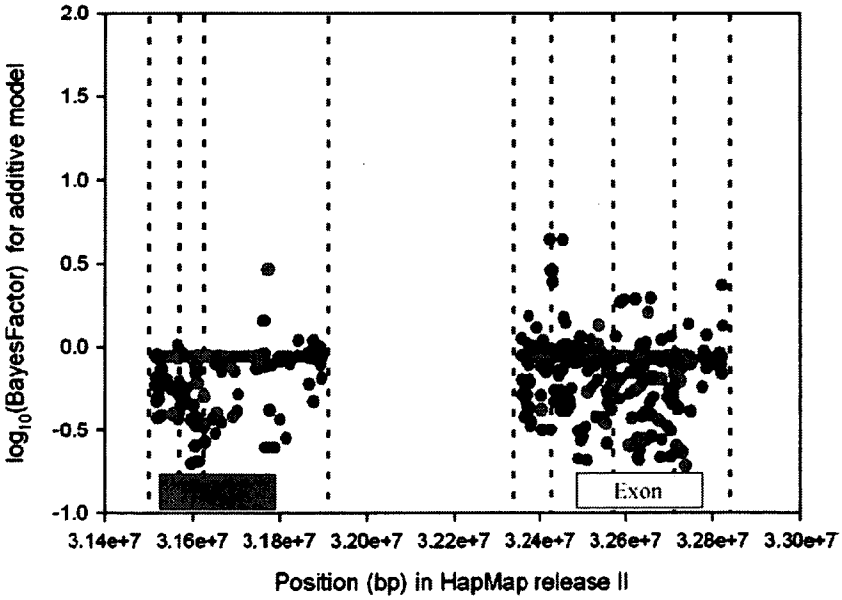


Fig. 1. Results of imputing SNP in the *NRG1* gene. The weights of evidence were calculated using imputed genotypes (red circles) and observed genotypes (black circles). Data from SNPs that constructed the significant haplotype in the first-set samples are shown in blue circles. Dotted lines indicate the estimated hot spots from the HapMap database. The SNP position from the HapMap release II database is plotted on the $X$ axis.

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Table 4
Confirmation analysis of significant haplotypes from first-set analysis

| Samples | SNPID | 1-window | 2-windows | 3-windows |
|---|---|---|---|---|
| Case–control | C#5 | .408 | .101 | |
| | C#6 | .362 | .601 | .120 |
| | C#7 | .371 | | |
| Family samples | C#5 | .107 | .323 | |
| | C#6 | .964 | .846 | .505 |
| | C#7 | .499 | | |
| Combined samples | C#5 | .976 | .591 | |
| | C#6 | .389 | .303 | .478 |
| | C#7 | .801 | | |

(if 1000 SNPs of 10,000,000 common human SNPs might be associated with a disease, we may assign a prior odds of association of 1/10,000. Therefore, a Bayes factor more than 10,000 (or $\log_{10}$ [Bayes factor] more than 4) is required (Balding, 2006)). Thus, these results indicate a low probability for association in our sample.

### 3.3. *Confirmation analysis of the positive haplotypes using different case–control samples and family samples*

To confirm the significance of exon region C#5–6–7 in the first-set samples, we conducted a confirmation analysis using independent case–control samples and family samples. In these analyses, we could not replicate this association. To increase the power, we combined samples (first-set and confirmation samples) but again we could not detect an association in this explorative analysis (Table 4).

## 4. Discussion

In the present study, using three large and independent samples, our data did not provide sufficient evidence for associations between tagging SNPs in the $HAP_{ICE}$ and exon regions of *NRG1* and schizophrenia in the Japanese population.

We could not replicate previous reports for the $HAP_{ICE}$ region (Stefansson et al., 2002; Stefansson et al., 2003); however, the results of this study are in concordance with our previous replication study in the Japanese population (schizophrenia=607, controls=515) (Iwata et al., 2004). Another study (Fukui et al., 2006), however, examined independent Japanese samples (belonging to one-third of confirmation case–control samples) and reported a positive association. Specifically, that study reported a significant association of haplotypes constructed by three core SNPs from Stefansson et al. (SNP8NRG221533 ($HAP_{ICE}$#3), SNP8NRG241930 ($HAP_{ICE}$#7) and SNP8NRG243177 ($HAP_{ICE}$#8)), and one more intronic SNP (rs1081062), as well as a trend for association of rs1081062. Since our tagging SNPs could not involve this SNP (rs1081062), we found by consulting the latest HapMap database (release#21a) that rs1081062 is tagged by rs13274954 ($HAP_{ICE}$#11); moreover, neither $HAP_{ICE}$#10 nor its haplotypes ($HAP_{ICE}$#3–7–8–11) were associated with schizophrenia (Global *P*-value= 0.540). Therefore, the aforementioned positive report could have been the result of type I error due to inadequate sample size (schizophrenia=349, controls=424) (Fukui et al., 2006). Or, as the authors speculated (Fukui et al., 2006), the different clinical backgrounds (e.g. genetic loading) in each sample could have led to inconsistent results. In this regard, a recent study reported that *DAOA/G30*, which is also a strong candidate gene for schizophrenia, influences susceptibility to the symptomatology of psychiatric disorders including schizophrenia and bipolar disorder, but not to diagnosis itself (Williams et al., 2006).

In the coding region, our results indicated the importance of controlling inflation of the type I error rate due to multiple testing, when a significant association is obtained in an analysis that involves several markers. In this study we found significant associations only from haplotype-wise analysis, not from allele- or genotype-wise analysis. It is generally accepted that a haplotype-wise analysis gives high power. At the same time, haplotype-wise analysis, especially multi-marker analysis or sliding-window analysis, tends to increase the chance of false positive results, since numerous hypotheses are examined. Bonferroni correction is typically used for solving multiple testing problems; however, since markers are not independent due to the existence of LD, Bonferroni correction is thought to be too conservative.

Therefore, we adopted two methods to validate the observed association; firstly, we imputed ungenotyped SNPs that might reflect a significant haplotype based on observations including our genotypic distribution of tagging SNPs and LD structure from the HapMap database. However, our simulation suggests that results for ungenotyped SNPs do not provide sufficient evidence for association. In other words, there was no SNP which could reflect a significant haplotype in the current data in HapMap release II. Secondly, we examined independent sets of samples for which a significant association was obtained in the initial screening analysis. We considered this to be the best strategy at present; however, the former significance of the exon region haplotype could not be replicated though independent case–control and family trios samples.

It is unlikely that negative results are due to type II error since a large sample size was used in this study; moreover, power analyses showed that the power was more than 80% when genotype relative risk (GRR) was set at 1.2–1.65 and 1.6–3.1 for confirmation case–

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control samples and family samples, respectively (MAF=2.4% and 47%), under a multiplicative model of inheritance in first-set screening samples.

Regarding interpretation of the results from this study, several limitations should be mentioned: Firstly, we did not screen the entire region of *NRG1*. On that point, Corvin et al. showed an independent 'at-risk' haplotype close to an EST cluster of unknown function (*Hs*.97362) within intron 1 of *NRG1* (Corvin et al., 2004). Secondly, our samples were not assessed with the use of the standard structured interview, and therefore have the possibility of false negatives due to misdiagnosis or sampling bias. Detailed association analyses with dense markers in the entire region of *NRG1* in well-phenotyped samples, including symptomatology, are essential in future study.

In conclusion, these results indicate that the positionally and functionally attractive regions of *NRG1* are unlikely to contribute to susceptibility to schizophrenia in the Japanese population. Moreover the nature of our results support that two-stage analysis with large sample size is appropriate to examine the susceptibility genes for common diseases; independent samples for examination of significance found in screening results should be an integral part of experimental design in genetic association analysis. Imputation methods should also be used when only haplotype association shows significance, in order to check for possible causal SNPs that can reflect the haplotype.

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**Contributors**

MI and NT designed the study, wrote the protocol and drafted the manuscript. MI, NT, SS, BA, YW, AN, YY, TK, YK, TK, and KK performed laboratory assays and the data-analysis. RH, HU, TI, TS, and MT advised on data-analysis. NO and NI participated in the design of the study, interpretation of the data, and drafting of the manuscript. All authors contributed to and have approved the final manuscript.

**Conflict of interest**

None.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2008.01.010.

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# Association analysis of AKT1 and schizophrenia in a UK case control sample

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**Abstract**

AKT1 (V-akt murine thyoma viral oncogene homolog 1) is involved in intracellular signalling pathways postulated as of aetiological importance in schizophrenia. Markers in the *AKT1* gene have also recently been associated with schizophrenia in two samples of European origin and in Japanese and Iranian samples. Aiming to replicate these findings, we examined ten SNPs spanning *AKT1* in a UK case-control sample (schizophrenia cases $n=673$, controls $n=716$). These included all SNPs previously reported to be associated in European, Japanese and Iranian samples, alone or in haplotypes, as well as additional markers defined by the Haploview Tagger program (pair-wise tagging, minimum $r^2=0.8$, minor allele frequency=0.02). We found no association with single markers (min $p=0.17$). We found weak evidence for association ($p=0.04$) with a four marker haplotype reported as significant in the original positive European sample of Emamian et al. [Emamian, E.S., Hall, D., Birnbaum, M.J., Karayiorgou, M., Gogos, J.A., 2004. Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. Nat. Genet. 36, 131–137] and also an overlapping three marker haplotype ($p=0.016$) that had previously been reported as significant in a Japanese sample. Nominal $p$-values for these haplotypes did not survive correction for multiple testing. Our study provides at best weak support for the hypothesis that *AKT1* is a susceptibility gene for schizophrenia. Examination of our own data and those of other groups leads us to conclude that overall, the evidence for association of *AKT1* as a susceptibility gene for schizophrenia is weakly positive, but not yet convincing.


*Keywords:* AKT1; Schizophrenia; Association; Candidate gene

## 1. Introduction

Emamian et al. (2004) proposed that alterations in brain protein kinase activity contribute to the aetiology of schizophrenia. In pursuit of this hypothesis, they examined the abundance of seven protein kinases in lymphoblast cell lines. Reduced AKT1 expression was found in cell lines derived from schizophrenic patients compared to controls, a finding subsequently confirmed in *post-mortem* frontal cortex and hippocampus. Moreover, they also found reduced phosphorylation of GSKβ3, a substrate of AKT1. These data provide a

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plausible case that AKT1 might be involved in the pathophysiology of schizophrenia, a hypothesis whose aetiological relevance they explored by genetic association using 5 SNPs spanning the gene (see Tables 1–3 for SNP nomenclature) in 268 US families of North European origin containing one or more individuals with schizophrenia. The initial evidence for association was weak but one marker, (SNP3), yielded evidence for association ($p=0.05$, uncorrected) as did a number of haplotypes (minimum $p=0.04$, corrected) each of which shared alleles T and C at SNPs 2 and 3 (Emamian et al., 2004), (referenced hereafter as the core haplotype). The core haplotype was also associated with reduced AKT1 protein expression in 20 control lymphoblast cell lines. Follow-up studies in three independent Japanese samples gave mixed results. Two studies consisted of over 500 cases and over 400 controls. The first (Ohtsuki et al., 2004) found no association (allelic or haplotypic) while the second (Ikeda et al., 2004) reported weak evidence for association with a different variant and different haplotype to that of Emamian et al. (2004) (Table 2), with allele C of the core being carried in haplotypes that were both over and underrepresented in cases. A third study in a Japanese sample of 124 families found no association (allelic or haplotypic) (Ide et al., 2006). Schwab et al. (2005) found significant association with 3 of 7 SNPs tested in *AKT1* in 79 sib pair families of German origin. The associated SNPs included SNP3, $p=0.027$, which was nominally significant in the Emamian study as well as two other SNPs, with the strongest result (SNP2a, rs10149779, $p=0.002$) remaining significant after correction for multiple testing ($p=0.014$). The most significant haplotype from the Emamian study (SNP2/SNP3/SNP4, TCG, Table 2) was also significantly over-represented in cases as was the TTA haplotype (formed by the same SNPs), which had been under-transmitted to cases in the study of Emamian et al. (2004) and which does not carry the core TC haplotype. Several other haplotypes created by various permutations of markers were also significantly over-transmitted with illness, with the strongest evidence coming from a haplotype derived from SNP1/SNP2a/SNP3 ($p=0.0013$ corrected for multiple testing), Table 2. For all haplotypes in which SNP2 was included, the over-transmitted haplotype carried Emamian's core T allele at SNP2 but the finding of the earlier study was not precisely recapitulated since haplotypes carrying either C or T at core SNP3 were significantly over-transmitted.

Further studies have been less supportive (Bajestan et al., 2006; Liu et al., 2006). The 5 SNPs genotyped by Emamian et al. (2004) were genotyped in 218 families from Taiwan (Liu et al., 2006) with no significant association from either single markers or haplotypes. The same SNPs were also typed in an Iranian case control sample, (schizophrenia cases $n=321$, controls $n=383$) (Bajestan et al., 2006). Again, neither the SNPs nor the

Table 1
Results: single markers

| SNP ID | Dist to next SNP (bp) | Base change allele 1/2 | Sample sized | Allele 1 count (freq) | Allele 2 count (freq) | *p*-value (1df) |
|---|---|---|---|---|---|---|
| rs3803300 (SNP1) | 4816 | G/A | Case ($N=660$) | 1194 (0.90) | 126 (0.10) | 0.40 |
| | | | Control ($N=707$) | 1292 (0.91) | 122 (0.09) | |
| rs2498784 (SNP1a) | 5229 | T/C | Case ($N=658$) | 102 (0.08) | 1214 (0.92) | 0.93 |
| | | | Control ($N=712$) | 109 (0.08) | 1315 (0.92) | |
| rs1130214 (SNP2) | 8648 | T/G | Case ($N=586$) | 361 (0.31) | 811 (0.69) | 0.73 |
| | | | Control ($N=660$) | 415 (0.31) | 905 (0.69) | |
| rs10149779 (SNP2a) | 4400 | A/G | Case ($N=658$) | 398 (0.30) | 918 (0.70) | 0.69 |
| | | | Control ($N=711$) | 440 (0.31) | 982 (0.69) | |
| rs2494738 | 279 | A/G | Case ($N=662$) | 109 (0.08) | 1215 (0.92) | 0.68 |
| | | | Control ($N=705$) | 110 (0.08) | 1300 (0.92) | |
| rs3730358 (SNP3) | 6513 | C/T | Case ($N=608$) | 1043 (0.86) | 173 (0.14) | 0.30 |
| | | | Control ($N=679$) | 1145 (0.84) | 213 (0.16) | |
| rs2498799 (SNP4) | 702 | G/A | Case ($N=592$) | 918 (0.78) | 266 (0.22) | 0.17 |
| | | | Control ($N=659$) | 991 (0.75) | 327 (0.25) | |
| rs2494732 (SNP5) | 46 | T/C | Case ($N=588$) | 652 (0.55) | 524 (0.45) | 0.80 |
| | | | Control ($N=663$) | 742 (0.56) | 584 (0.44) | |
| rs3803304 | 6051 | C/G | Case ($N=653$) | 327 (0.25) | 979 (0.75) | 0.56 |
| | | | Control ($N=707$) | 368 (0.26) | 1046 (0.74) | |
| rs2498804 (SNP A) | – | G/T | Case ($N=660$) | 911 (0.69) | 409 (0.31) | 0.46 |
| | | | Control ($N=709$) | 960 (0.68) | 458 (0.32) | |

Allele counts, frequencies and *p*-values across *AKT1* locus. SNP ID includes both rs no. and ID used in Emamian et al. (2004), Ikeda et al. (2004) and Schwab et al. (2005).

Table 2
Comparison of associated haplotypes

| Study | Population | rs3803300 (SNP1) | rs2498784 (SNP1a) | rs1130214 (SNP2) | rs10149779 (SNP2a) | rs2494738 | rs3730358 (SNP3) | rs2498799 (SNP4) | rs2494732 (SNP5) | rs3803304 | rs2498804 (SNPA) | SCZ | CON | *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Emamian et al. | US | | | T | | | C* | G* | | | | – | 0.15 | 0.0006 |
| Schwab et al. | German | | | T | | | C* | G* | | | | 0.17 | 0.10 | 0.023 |
| This study | UK | | | T | | | C | G | | | | 0.19 | 0.17 | 0.37 |
| Schwab et al. | German | G* | | | T | | C* | | | | | 0.17 | 0.09 | 0.0013 |
| This study | UK | G | | | T | | C | | | | | 0.18 | 0.16 | 0.51 |
| Emamian et al. | US | | | T | | | C* | G* | G | | | – | – | 0.004 |
| This study | UK | | | T | | | C* | G* | G | | | 0.13 | 0.10 | 0.04 |
| Schwab et al. | German | | | T | | | C | G | G | | | 0.10 | 0.07 | 0.11[a] |
| Ikeda et al. | Japanese | | | T | | | C | G | G | | | 0.02 | 0.01 | 0.18[a] |
| Ikeda et al. | Japanese | | | | | | C* | G* | G | | | 0.32 | 0.27 | 0.014 |
| This study | UK | | | | | | C* | G* | G | | | 0.22 | 0.17 | 0.016 |
| Bajestan et al. | Iranian | A | | G* | | | C* | A | G | | | 0.07 | 0.03 | 0.004 |
| This study | UK | A | | G | | | C | A | G | | | 0.05 | 0.05 | 0.73 |
| This study | UK | A | | | | | C* | G* | | | | 0.04 | 0.02 | 0.006 |
| Ikeda et al. | Japanese | | | | | | C* | G* | A | | | 0.17 | 0.24 | 0.0001 |
| This study | UK | | | | | | C | G | A | | | 0.55 | 0.56 | 0.55 |

Comparison of the most significant *p*-values from current studies reporting positive association with *AKT1* and schizophrenia. Significant haplotypes are marked in grey.
[a]Personal communications, *Ancestral allele in NCBI entrez SNP.

haplotypes from the Emamian study were associated. However, a novel five marker haplotype comprised of SNPs1-5, showed some evidence for association (global $p=0.05$ uncorrected) with haplotype AGCAG being more frequent in cases compared to controls (uncorrected $p=0.004$, Bonferroni corrected, $p=0.03$, case freq 0.068, control freq 0.034). Given the diverse range of ethnicities studied so far, lack of consistency of the patterns of association between studies is potentially explicable in terms of population differences in LD and modest power to detect weak genetic effects. Moreover, in the light of partial replication of the original findings at the level of a specific haplotype in the only other European origin sample so far reported, *AKT1* is clearly worth further investigation in other samples of broadly similar ethnicity.

We set out to investigate *AKT1* in schizophrenia using a moderately large UK based case control sample under the following strategies. We genotyped SNPs 1–5 from Emamian et al. (2004), and additional markers

Table 3
LD data for control sample

| | rs3803300 (SNP1) | rs2498784 (SNP1a) | rs1130214 (SNP2) | rs10149779 (SNP2a) | rs2494738 | rs3730358 (SNP3) | rs2498799 (SNP4) | rs2494732 (SNP5) | rs3803304 | rs2498804 (SNPA) |
|---|---|---|---|---|---|---|---|---|---|---|
| rs3803300 (SNP1) | x | 0.86 | 0.01 | 0.01 | 0.46 | 0.01 | 0.11 | 0.07 | 0.00 | 0.12 |
| rs2498784 (SNP1a) | 0.98 | x | 0.02 | 0.02 | 0.38 | 0.00 | 0.11 | 0.06 | 0.00 | 0.10 |
| rs1130214 (SNP2) | 0.36 | 0.78 | x | 0.95 | 0.00 | 0.25 | 0.06 | 0.21 | 0.11 | 0.06 |
| rs10149779 (SNP2a) | 0.41 | 0.81 | 0.99 | x | 0.01 | 0.26 | 0.06 | 0.20 | 0.12 | 0.07 |
| rs2494738 | 0.71 | 0.62 | 0.30 | 0.36 | x | 0.02 | 0.12 | 0.09 | 0.00 | 0.16 |
| rs3730358 (SNP3) | 0.63 | 0.55 | 0.80 | 0.80 | 1 | x | 0.40 | 0.22 | 0.49 | 0.37 |
| rs2498799 (SNP4) | 0.63 | 0.70 | 0.29 | 0.30 | 0.71 | 0.85 | x | 0.43 | 0.40 | 0.69 |
| rs2494732 (SNP5) | 0.77 | 0.79 | 0.60 | 0.59 | 0.92 | 0.98 | 1 | x | 0.44 | 0.59 |
| rs3803304 | 0.05 | 0.18 | 0.37 | 0.38 | 0.14 | 0.96 | 0.65 | 1 | x | 0.69 |
| rs2498804 (SNPA) | 0.79 | 0.76 | 0.25 | 0.27 | 0.95 | 0.96 | 0.99 | 1 | 0.97 | x |

LD data for control sample. $D'$ is below diagonal and $r^2$ is above diagonal.

reported by others, SNP1a, SNP2a, (Schwab et al., 2005) and SNPA (Ikeda et al., 2004). We specifically tested all significant associated haplotypes reported by Emamian et al. (2004), ($n$=7), Ikeda et al. (2004), ($n$=9), Schwab et al. (2005), ($n$=23) and the Iranian 5 marker haplotype (Bajestan et al., 2006), (a total of 30 tests), although our primary hypothesis concerned the European origin haplotypes ($n$=28). Additionally, we derived tagged SNPs across the *AKT1* locus after genotyping all the above markers in the CEU panel used by the HapMap project and combining those data with all additional markers available in the HapMap (version 1.65) and performed two and three marker haplotype analyses for all marker combinations.

## 2. Materials and methods

### 2.1. Subjects

All case-control subjects used in this study were unrelated Caucasians born in the UK or Ireland. All cases met DSM-IV criteria for schizophrenia. Consensus diagnoses were made by two raters from all available information following a semi-structured interview, SCAN or PSE (Wing et al., 1974, 1990), and examination of case notes. The cases consisted of 456 males and 217 females, average age at collection 44.5 years ±14.6, whilst the controls consisted of 482 males and 234 females, average age at collection 41.5 years ± 11.5 years. Control individuals were group matched to cases for age, sex, and ethnicity from more than 1400 blood donors recruited from the National Blood Transfusion Service. Individuals on medication are not allowed to donate blood in the UK nor are they remunerated even for expenses. Thus unlike in some countries, donating blood in the UK is entirely an altruistic process that does not tend to enrich for indigents, or people with substance abuse or psychosis. Donors were not screened for the absence of psychiatric illness, as this does not affect the power when a disease has the population prevalence of schizophrenia (Owen et al., 1997). Multicentre and Local Research Ethics Committee approval was obtained, and all subjects, both cases and controls, gave written informed consent to participate. We previously found no evidence for population stratification within the samples based on the distribution of $p$-values obtained from genotyping pooled samples for >300 SNPs (Williams et al., 2005a). We also tested for evidence of substructure in approximately one-third of our sample with STRUCTURE (Pritchard et al., 2000) by using 97 SNPs scattered across the genome and 1000 SNPs targeted to regions on chromosomes 10 and 22 (Georgieva et al., 2006).

Sample power was estimated to be 80% for the "core TC haplotype" given our observed frequency, an OR of 1.3, alpha=0.05 and 79% for the associated TCG haplotype in Table 2, under the same parameters. For rs3730358 (associated in both Emamian et al., 2004 and Schwab et al., 2005), we estimated power to be 73% given an OR of 1.3, alpha=0.05.

### 2.2. SNP selection

We initially selected for genotyping, SNPs 1–5 from Emamian et al. (2004), (rs3803300, rs1130214, rs3730358, rs2498799, rs2494732 respectively), two additional SNPs from Schwab et al. (2005), (rs2498784 and rs10149779, SNP1a and SNP2a respectively), and 1 additional SNP from Ikeda et al. (2004), (rs2498804, SNPA), in order to be able to test the specific marker and haplotype hypotheses generated by those studies. All SNPs were optimised on the same CEPH DNA samples used in the international HapMap project for purposes of both error checking (all genotypes were checked against HapMap data for concordance) and also for tag SNP selection. We used our CEPH data and all other available CEPH data from the HapMap release 16C.1 June 2005 (Generic genome browser version 1.65) across the *AKT1 locus* from UCSC May2004 chr14:104304140–104341530 (including 8.4 kb sequence upstream and 2.6 kb sequence downstream of *AKT1*) and performed pairwise analysis with TAGGER as implemented in Haploview (Barrett et al., 2005) using settings $r^2$>0.8, minimum MAF 2%. This suggested as additional tagging SNPs rs2494738 and rs3803304, none of which have been genotyped in previous *AKT1* association studies (Emamian et al., 2004; Ohtsuki et al., 2004; Ikeda et al., 2004; Schwab et al., 2005; Liu et al., 2006; Bajestan et al., 2006; Ide et al., 2006).

### 2.3. Genotyping

8/10 SNPs were genotyped using the Sequenom MassARRAY™ system as per the manufacturer's instructions with either hME or iplex chemistries. SNPs 1a and 2a were genotyped with allele-specific PCR using the Amplifluor system (Myakishev et al., 2001; Hawskins et al., 2002).

Assay design and PCR conditions are available on request. All assays used to type the full association sample were optimised initially by genotyping DNA from 30 CEPH parent–offspring trios from 21 families (Utah residents with ancestry from northern and western Europe), as detailed in the international HapMap project

(The International HapMap Consortium, 2005). We re-genotyped 46 of these samples along with the case control sample to provide a measure of genotyping accuracy. All genotypes were called blind to sample identity and affected status.

### 2.4. Statistical analysis

Tests of genotypic and allelic association were performed using contingency tables. Haplotype analyses were performed using the EM algorithm and a permutation test as implemented in program EH plus (Zhao et al., 2000) for global significance. Association of specific haplotypes was estimated with Cocaphase (Dudbridge, 2003). LD values were calculated using the ldmax program within the GOLD software (Abecasis and Cookson, 2000).

## 3. Results

Genotype data for SNP2, rs2494738, rs3803304 and SNPA from our assays in the same 90 CEPH DNA samples used in the International HapMap Project were 100% concordant with HapMap data. 100% concordance was also achieved between genotype data of 46 CEPH DNA samples typed in our initial assay optimisation stage and the same samples contained within our case control sample set for all 10 SNPs.

Genotype data were in Hardy Weinberg equilibrium for both cases and controls for all SNPs. No significant differences between cases and controls were observed for any single markers by allele (Table 1) or genotype (data not shown).

We specifically tested those marker combinations reported to yield the most significantly associated haplotypes by Emamian et al. (2004) including the core haplotype, (SNP2/SNP3, TC), those of Schwab et al. (2005), (SNP1/SNP2a/SNP3, GTC) and Ikeda et al. (2004), (SNP3/SNP4/SNP5, CGG and CGA as well as seven other overlapping haplotypes also significantly associated in the Japanese sample). Table 2 summarizes the most significant haplotypes with the ancestral alleles marked as *. Marker combination SNP2/SNP3/SNP4 which gave the most significant results in the Emamian study, gave a global $p$-value of 0.08 in our sample. However, the TCG haplotype for this marker combination which gave nominal significance in the initial Emamian study ($p=0.0006$) and also in that of Schwab et al. (2005), ($p=0.02$) was not significantly associated in our sample, $p=0.37$, although a non significant trend was observed in the previously reported direction (Table 2). Our case and control frequencies for this haplotype were 0.19 and 0.17 respectively, compared to 0.17 and 0.10 in the German sample (Schwab et al., 2005) and 0.15 in the US sample of European origin of Emamian et al. (2004).

Global haplotype analysis of markers (SNP2/SNP3) forming the core two-marker haplotype of Emamian et al. (2004) revealed no significant evidence for association (global $p=0.09$) nor did specific analysis of the TC phased core haplotype ($p=0.41$). However, a specific phased 4 marker haplotype (SNP2/SNP3/SNP4/SNP5, TCGG, Table 2) which was also significant in the Emamian study, $p=0.004$, was associated in our sample, $p=0.04$ (case freq=0.13, control freq=0.10). The same haplotype was not significantly associated in either the German (Schwab et al., 2005) or in the Japanese (Ikeda et al., 2004) samples, ($p=0.11$ and 0.18 respectively), (personal communications).

The most significant haplotype of (SNP1/SNP2a/SNP3 GTC, Table 2) reported by Schwab et al. (2005) was not associated in our sample, ($p=0.51$), although the trend with frequencies of 0.18 and 0.16 in our cases and controls respectively, compared to 0.17 and 0.09 in the German sample was in the same direction.

Global haplotype analysis of SNP3/SNP4/SNP5 which was the most significant haplotype in the Japanese study of Ikeda et al. (2004) was significantly associated (uncorrected $p=0.04$) in our sample. The largest difference in haplotype frequency for this combination was 5% for the haplotype CGG (Table 2, $p=0.016$). The same haplotype was also significantly associated in the same direction in the Japanese sample (Ikeda et al., 2004), $p=0.014$, although frequencies in the samples are different (CGG=0.27 in Japanese controls vs. 0.17 in Cardiff controls). The most significant haplotype associated in the Japanese sample was SNP3/SNP4/SNP5 CGA, ($p=0.0001$), (Ikeda et al., 2004). This was not significantly associated in our sample. The haplotype frequencies in our sample and in that of Ikeda et al. (2004) are substantially different (Table 2). Ikeda et al. (2004) also reported 7 overlapping haplotypes with individual $p$-values less than 0.05. None of these was significantly associated in our sample.

Although we selected the markers predicated on single locus (i.e. pairwise tagging), in order to try to capture the effect of unknown variants that are not represented in HapMap, we undertook 2 and 3 marker haplotype analysis across all the markers including those additional SNPs recommended by our tagging procedure. We obtained evidence for association for haplotypes of SNP1/SNP3/SNP4, global $p=0.04$, which overlaps physically with the most significant haplotypes reported by Schwab et al. (2005) and Emamian et al. (2004). On closer inspection, the effect came from two haplotypes with frequencies of

less than 5%, (GTG case = 0.009, control = 0.018, $p = 0.06$ and ACG, case = 0.043, control = 0.023, $p = 0.006$). Allele C of SNP3 is common to our significant haplotype (SNP1/SNP3/SNP4, ACG) and the most significant haplotypes of Emamian et al. (2004) and Schwab et al. (2005), whilst allele G of SNP4 is common to our SNP1/SNP3/SNP4, ACG haplotype and the SNP2/SNP3/SNP4, TCG haplotype of Emamian et al. (2004) and Schwab et al. (2005).

## 4. Discussion

Following the initial report (Emamian et al., 2004) and mixed replication evidence (Ohtsuki et al., 2004; Ikeda et al., 2004; Ide et al., 2006; Schwab et al., 2005; Liu et al., 2006; Bajestan et al., 2006) we sought to provide further evidence for association between schizophrenia and polymorphisms in *AKT1*. The question of when the evidence for association between disease and gene is convincing is a vexing one for several reasons. Ideally, such evidence would come from repeated demonstration of a directional association (even if not significant) between a disorder and a specified allele such that pooled or meta-analyses demonstrates a clear highly significant directional effect. However, when based upon indirect association, replication of specified alleles may not be easily obtained due to a mixture of population differences in allelic heterogeneity at the locus, patterns of LD, allele frequencies, phenotypic variation relevant to the associated allele, or exposure to environmental variables with which a risk allele interacts (O'Donovan and Owen, 1999). Moreover, mathematical analyses show that where the true effect size of a susceptibility allele is weak, opposite alleles may be genuinely associated with disease even in populations with similar LD measures, allele frequencies, and identical effect sizes at the functional locus (Moskvina and O'Donovan, in press). Given the above, while association to the same allele across studies should at least be sought, it cannot be a prerequisite for considering a study as supportive of association between disease and a gene. Instead, we believe it is legitimate to view association to any allele or haplotype that both survives honest appropriate correction for multiple testing and is based on a well-designed quality-controlled study as significant evidence for replication at the gene-level. When multiple studies meet this criterion, as we consider to be the case for example for dysbindin (Williams et al., 2005b), then in our view, the evidence can be considered convincing.

Our single marker data for AKT1 provide no evidence for association with schizophrenia, but haplotype analysis showed some trends similar to the existing data, albeit, none that remain significantly associated in the context of multiple testing. When associated haplotypes from all of these studies are aligned (Table 2), it is apparent that the most significant risk haplotypes across studies overlap, making the correction for multiple testing over conservative. (Of the $p$-values reported in Table 2, the most significant haplotype of Schwab et al. (2005) is already corrected for multiple testing by simulation (Becker and Knapp, 2004). The most significant haplotype in the Emamian study ($p = 0.006$) is uncorrected, but remains significant after adjustment for the comparisons made in the study, ($p < 0.04$).

In Table 2 where we show the most significant haplotype reported from each study and compare these specific haplotypes across the published data and with our own data, the SNP3/4, alleles CG combination occurs in 6/7 of the significant haplotypes. This trend also extends to the Japanese sample (Ikeda et al., 2004), but not in the Iranian sample (Bajestan et al., 2006) where the only significant haplotype contains the A allele at SNP 4. Also, when all ethnicities are considered together, a trend for a longer overlap is observed with SNPs 3/4/5, CGG in the significant risk haplotypes of both this, the initial positive and the Japanese study, (4/10 significant haplotypes). However, given that alleles C and G are respectively the major alleles at SNP3 and SNP4, and are present on numerically more haplotypes than SNP3 allele T, this may simply be chance rather than a reflection of a genuine pattern in the data. Nevertheless, similar directions of effect were observed in our sample for the haplotypes most significantly over-represented in samples of European origin. Further confidence could be achieved if the four marker haplotype (SNP2/SNP3/SNP4/SNP5, TCGG) which was associated in the original positive study and in our own data was also significant in the German study and Japanese samples, particularly since (SNP3/SNP4/SNP5, CGG) was also significant in the Japanese sample. However, the TCGG haplotype was not significantly associated in the German sample, $p = 0.11$ (personal communication) although the trend was in the same direction, and the same haplotype was of low frequency in the Japanese sample of Ikeda et al. (2004), (personal communication) and was not significant in the Japanese sample of Ohtsuki et al. (2004), (personal communication).

Our haplotype frequencies were broadly similar to those other samples of European, origin (Emamian et al., 2004; Schwab et al., 2005), although when compared to the German sample (Schwab et al., 2005), both our case and control frequencies were more similar to the German cases than to the German controls (Table 2). Comparison of LD patterns across studies showed $D'$

values for our sample to be very similar to those of Emamian et al. (2004) and also to those of Schwab et al. (2005) and the CEPH sample (Table 3). Schwab et al. (2005) also suggested increased recombination in the region between SNP2a and SNP3 based on a decrease in $D'$ in this region compared to neighbouring regions. By genotyping all markers in the same CEPH individuals used in the international HapMap project, our data agree with this finding (data not shown).

### 4.1. Conclusions

In a bid to replicate association of *AKT1* with schizophrenia, we genotyped those polymorphisms that provided evidence for association in samples of both European and Japanese origin and also undertook a pairwise analysis across the locus in a large UK based case control sample. None of our findings survive correction for even modest degrees of multiple testing, and therefore we must conclude that our study does not provide robust support for the hypothesis. However, in our sample, we find (uncorrected) support for a phased 4 marker haplotype that was significantly associated in the original positive association study (Emamian et al., 2004) and which also shows non-significant trends in the same direction in the only other sample of white European origin (Schwab et al., 2005), ($p$=0.11, 2-tailed) and, notwithstanding a very low frequency, in the positive Japanese study (Ikeda et al., 2004), ($p$= 0.18). This phased haplotype also shares 3 alleles with a significant phased 3 marker haplotype in a Japanese sample (Ikeda et al., 2004). Thus, while our study does not provide strong support, the current evidence for association between *AKT1* across different studies remains intriguing, and worthy of further investigation.

## 5. Contributors

NN, HW, SD, LC, TP performed laboratory assays. NN performed the data-analysis and drafted the manuscript. VM and RS advised on data-analysis. NW participated in the design of the study. IN was responsible for data-management. MI and NI provided haplotypic data and analysis from an independent sample. MOD and MJO participated in the design of the study, interpretation of the data, and drafting of the manuscript. All authors read and approved the final manuscript.

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## 7. Conflicts of interest

The author(s) declare that they have no conflicts of interest.

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Development/Plasticity/Repair

# Brain-Derived Neurotrophic Factor Regulates Cholesterol Metabolism for Synapse Development

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**Brain-derived neurotrophic factor (BDNF) exerts multiple biological functions in the CNS. Although BDNF can control transcription and protein synthesis, it still remains open to question whether BDNF regulates lipid biosynthesis. Here we show that BDNF elicits cholesterol biosynthesis in cultured cortical and hippocampal neurons. Importantly, BDNF elicited cholesterol synthesis in neurons, but not in glial cells. Quantitative reverse transcriptase-PCR revealed that BDNF stimulated the transcription of enzymes in the cholesterol biosynthetic pathway. BDNF-induced cholesterol increases were blocked by specific inhibitors of cholesterol synthesis, mevastatin and zaragozic acid, suggesting that BDNF stimulates *de novo* synthesis of cholesterol rather than the incorporation of extracellular cholesterol. Because cholesterol is a major component of lipid rafts, we investigated whether BDNF would increase the cholesterol content in lipid rafts or nonraft membrane domains. Interestingly, the BDNF-mediated increase in cholesterol occurred in rafts, but not in nonrafts, suggesting that BDNF promotes the development of neuronal lipid rafts. Consistent with this notion, BDNF raised the level of the lipid raft marker protein caveolin-2 in rafts. Remarkably, BDNF increased the levels of presynaptic proteins in lipid rafts, but not in nonrafts. An electrophysiological study revealed that BDNF-dependent cholesterol biosynthesis plays an important role for the development of a readily releasable pool of synaptic vesicles. Together, these results suggest a novel role for BDNF in cholesterol metabolism and synapse development.**

*Key words:* **brain-derived neurotrophic factor; TrkB receptor; cholesterol; synapse development; lipid rafts; CNS neurons**

## Introduction

Among neurotrophins, brain-derived neurotrophic factor (BDNF) is highly expressed in brain and mediates multiple effects on CNS neurons (Bibel and Barde, 2000). One of its remarkable functions is the regulation of synaptic function and development (Poo, 2001). BDNF facilitates long-term potentiation (Figurov et al., 1996; Patterson et al., 1996) and excitatory synaptic transmission by enhancing transmitter release (Lessmann, 1998). Long-term treatment with BDNF increases the number of docked vesicles at synapses and the amount of synaptic vesicle proteins (Takei et al., 1997; Tartaglia et al., 2001). BDNF also induces complex effects on the dendritic arborization of pyramidal neurons (McAllister et al., 1995).

Responses to BDNF are mediated by a tyrosine receptor kinase B, TrkB. Binding of BDNF rapidly activates TrkB activity, which in turn triggers multiple intracellular signaling pathways (Reichardt, 2006). The downstream effectors include well characterized signal molecules, such as mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and phospholipase C-γ. However, how and where BDNF mediates its diverse biological actions remain poorly understood. One intriguing idea is that the distinct signaling or functions of BDNF are mediated in different subcellular compartments. Cholesterol-rich microdomains, called lipid rafts, have been proposed to provide a signaling platform for neurotrophic factor signaling (Simons and Toomre, 2000; Paratcha and Ibanez, 2002). We have shown that lipid rafts are required for BDNF-induced synaptic modulation (Suzuki et al., 2004) and chemotrophic guidance of nerve growth cones (Guirland et al., 2004). Very recently, it was shown that a tyrosine kinase Fyn determines the localization of TrkB in rafts (Pereira and Chao, 2007).

There is also increasing evidence that cholesterol itself is a crucial component of synaptic structure and function. Freeze-fracture electron microscopy and biochemical studies have shown that cholesterol is enriched in presynaptic terminals (Pfrieger, 2003; Takamori et al., 2006). Pharmacological depletion of cholesterol reduced BDNF-dependent synaptic transmission (Suzuki et al., 2004). In contrast, glial cell-derived cholesterol

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increased the efficacy of synaptic transmission (Mauch et al., 2001). More recently, suppression of the mevalonate pathway was shown to cause defects in learning and memory (Kotti et al., 2006), indicating the importance of cholesterol for the CNS.

Despite these studies, the regulation of cholesterol biosynthesis and metabolism in neurons remains ill-defined. The rate-limiting enzyme of cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, is expressed in hippocampal neurons (Krijnse-Locker et al., 1995), and radiolabeled cholesterol precursors are converted rapidly into cholesterol in cultured neurons (Saito et al., 1987; Vance et al., 1994). Although these reports indicate that cholesterol content and distribution are controlled in neurons, the actors regulating cholesterol biosynthesis in neurons remain to be identified.

Previously, we demonstrated that both BDNF and cholesterol increase dramatically during cortical development (Suzuki et al., 2004), suggesting that BDNF may regulate cholesterol biosynthesis in the brain. Here we investigate the effect of BDNF on cholesterol biosynthesis in cultured cortical and hippocampal neurons. The results suggest that BDNF elicits cholesterol biosynthesis for the development of presynaptic functions in the CNS.

## Materials and Methods

BDNF was a generous gift from Sumitomo Pharmaceuticals (Osaka, Japan). BDNF was dissolved at 2 mg/ml in PBS containing 1% bovine serum albumin (BSA) and was added to the culture medium at various concentrations for 0–5 d, as indicated. The following antibodies were used in this study. For Western blot analysis anti-synaptophysin (1:1000 dilution; Roche Diagnostics, Indianapolis, IN), anti-soluble *N*-ethylmaleimide-sensitive factor attachment protein-25 (anti-SNAP-25; 1:2000; Synaptic Systems, Göttingen, Germany), anti-caveolin-2 (1:1000; BD Transduction Laboratories, San Jose, CA), anti-neural tubulin (1:5000; Berkeley Antibody Company, Richmond, CA), anti-syntaxin (1:10000; Sigma, St. Louis, MO), anti-transferrin receptor (1:1000; Zymed, San Francisco, CA), anti-phospho-Trk (Y490; 1:1000; Cell Signaling Technology, Beverly, MA), and anti-TrkB (1:1000; BD Transduction Laboratories) were used. The anti-mouse IgG and anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch (West Grove, PA) (1:1000). For immunocytochemistry anti-TrkB (1:10; 794, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-synaptophysin (1:200; Santa Cruz Biotechnology), anti-microtubule-associated protein-2 (anti-MAP-2; 1:400; Millipore, Billerica, MA), anti-O4 (1:200; Millipore), and anti-glial fibrillary acidic protein (GFAP; 1:100; Sigma) were used. The anti-mouse IgG, anti-rabbit IgG, and anti-chick IgY secondary antibodies conjugated to Alexa 568 or Alexa 647 were purchased from Invitrogen (Carlsbad, CA) (1:200), and the antibodies conjugated to FITC were purchased from Jackson ImmunoResearch (1:400).

*Primary cultures.* For biochemical experiments the primary cultures of cerebral cortex and hippocampus were prepared from embryonic day 20 rats as described previously (Suzuki et al., 2002). This procedure was strictly in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology, Japan. Briefly, cerebral cortices or hippocampi were cut into small pieces and incubated at 37°C for 15 min in papain solution. Cells were separated by 5–10 gentle trituration passes, using a 10 ml measuring pipette, and were passed through a 70 μm cell strainer (Falcon, Oxnard, CA) to remove large debris. Cells were plated on polyethyleneimine-coated plates at $5 \times 10^4$ cells/cm$^2$ for cortical culture or $3 \times 10^4$ cells/cm$^2$ for hippocampal culture and were cultured in medium consisting of 5% heat-inactivated fetal bovine serum (FBS), 5% heat-inactivated horse serum, and 90% DMEM. Cultures were maintained in an incubator with 10% $CO_2$. After 3–4 d *in vitro* (3–4 DIV) the culture medium was changed to Neurobasal medium (Invitrogen) containing the B27 supplement and glutamine (Invitrogen) as well as the indicated concentrations of BDNF or other drugs as indicated. This preparation also was used for measuring soma size.

For neuron-rich cultures the plated cells were maintained in the presence of 10 μM cytosine 1-b-D-arabinofuranoside (Ara-C) until the assays were performed. The population of contaminating non-neuronal cells was determined by immunocytochemistry (see Fig. 3).

Astrocytes were prepared according to a previous report (Ohira et al., 2005). Briefly, dissociated cortical cells were seeded at the density of $5 \times 10^3$ cells/cm$^2$ and grown in flasks containing 10% FBS and 90% DMEM for >2 weeks. After reaching confluence, the cells were washed twice with PBS, harvested with 0.25% trypsin and 1 mM EDTA, and then replated in a new flask after a twofold dilution was performed. These procedures were repeated until MAP-2-positive neurons disappeared. Purified astrocytes were plated into six-well plates at a density of $3 \times 10^5$ cells/well. The indicated assays were performed after the cultures reached semi-confluence.

For electrophysiology the CA1–CA3 regions of hippocampi were isolated from postnatal day 0–1 rats and after dissociation. The cells were plated at a density of $3 \times 10^4$ cells/cm$^2$, as described (Nakayama et al., 2005). For the first 3 d the cells were grown in a mixed medium (1:1) containing B27-supplemented Neurobasal medium and 10% FBS-containing DMEM. Then, the medium was changed to Neurobasal medium containing 3 μM Ara-C to prevent proliferation of glial cells. Cells were treated with the indicated drugs from 3–4 to 6–7 DIV.

*Cholesterol measurement and cholesterol depletion.* After 3–5 d of treatment with the indicated drugs the cells were rinsed with PBS and lysed with lipid lysis buffer [containing the following (in mM): 50 Tris-HCl, pH 7.4, 1 EDTA, 150 NaCl, 10 NaF, 10 $Na_2P_2O_7$, plus 1% Triton X-100, 100 μM phenylarsine oxide, and protease inhibitor mixture]. Lysates were sonicated, and cellular cholesterol content was determined by using a total cholesterol measurement kit (Wako, Osaka, Japan), which uses an enzyme-coupled reaction in which cholesterol oxidase generates $H_2O_2$ from cholesterol and peroxidase catalyzes the reaction of $H_2O_2$ with 4-aminoantipyrine to yield a colored product. To estimate total cholesterol levels, we included cholesterol ester hydrolase. Cholesterol was depleted by the addition of 10 μM mevastatin (HMG-CoA reductase inhibitor; Sigma) or 100 μM zaragozic acid (squalene synthase inhibitor; Sigma) in the presence or absence of BDNF.

*Lipid analysis.* Sterol extraction was performed as based on the method of Tacer et al. (2002). Cortical neurons ($3 \times 10^6$ cells/100 mm Petri dish) and were treated with or without BDNF for 5 d. Cells were homogenized in PBS, and the homogenate was extracted in a 3.6-fold volume of isopropanol/*n*-heptane (1:3; v/v) by being vortexed for 30 s. The solution was added to 0.3 M $NaH_2PO_4$, pH 1.0, and sterols were extracted with constant shaking (300 rpm, for 30 min). After centrifugation (9100 × *g* for 15 min), the organic phase was recovered and dried by using a speed vacuum concentrator. Sterols were dissolved in isopropanol and resolved by thin-layer chromatography (TLC) on silica gel 60 TLC plates in hexane/ethyl acetate (3:1; v/v). Sterols were detected by using anisaldehyde solution [*p*-methoxy-benzaldehyde/acetic acid/$H_2SO_4$/ethanol; 0.93/0.38/1.25/34 (v/v)]. For densitometric analysis of the bands (see Fig. 1*E*,*F*), a CS analyzer version 2.0 (Atto, Tokyo, Japan) was used.

*Preparation of lipid rafts.* Raft fractionation was performed as described previously (Suzuki et al., 2004). All steps were performed with ice-cold reagents at 4°C. Briefly, cultured cortical neurons ($3 \times 10^6$ cells/100 mm Petri dish) were washed with PBS and homogenized in 500 μl of Triton lysis buffer [containing the following (in mM): 50 Tris-HCl, pH 7.4, 1 EDTA, 150 NaCl, 10 NaF, 1 $Na_3VO_4$, 10 $Na_2P_2O_7$, plus 1% Triton X-100, 100 μM phenylarsine oxide, protease inhibitor mixture, and protein phosphatase inhibitor mixture]. The lysates were incubated for 30 min, passed 10 times through a 25-G needle, and then mixed with an equal volume of 100% (w/v) sucrose in buffer A [containing the following (in mM): 50 Tris-HCl, pH 7.4, 5 NaCl, 1 $Na_3VO_4$, plus 100 μM phenylarsine oxide, protease inhibitor mixture, and protein phosphatase inhibitor mixture]. Then 1 ml of the sucrose–lysate mixture was transferred to a centrifuge tube (50 Ultra-Clear Tubes, 14 × 89 mm; Beckman Coulter, Palo Alto, CA) and overlaid sequentially with 8 ml of 35% (w/v) sucrose in buffer A, followed by 3.5 ml of 5% (w/v) sucrose in buffer A. After centrifugation in a Beckman Coulter SW41 rotor for 13 h at 200,000 × *g* at 4°C, the fractions were collected from the top of the gradient. The volume of the first fraction (fraction 1) was 2.5 ml, and the

volumes of the remaining fractions (fractions 2–6) were 2.0 ml. Successful fractionation was confirmed by Western blot analysis, using antibodies against raft and nonraft marker proteins (see Fig. 5A).

*Western blot analysis.* Total lysates were prepared as reported previously (Suzuki et al., 2002). Samples were sonicated with an equal volume of SDS sample buffer [0.125 M Tris-HCl, pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS, and 10% (v/v) 2-mercaptoethanol] and then heated at 100°C for 3 min and separated by SDS-PAGE (Suzuki et al., 2004). Samples were transferred to polyvinylidene fluoride membranes (Immobilon P membrane; Millipore). Membranes were blocked in Tris-buffered saline containing 0.2% Tween 20 (TBST) and 5% BSA, incubated with the indicated primary antibody in TBST containing 0.5% BSA (TBSTB) at room temperature for 90 min, and then washed three times with TBST. Membranes then were incubated with peroxidase-conjugated secondary antibodies in TBSTB at room temperature for 30 min and washed three times with TBST; a signal was detected by using ImmunoStar reagents (Wako).

*Filipin staining and immunocytochemistry.* Cortical neurons were fixed with 4% paraformaldehyde at room temperature for 3 h, rinsed three times with PBS, and blocked with 3% BSA in PBS (PBSB) for 30 min. For immunocytochemistry the cells were permeabilized with 0.2% Triton X-100 in PBS for 2 min and then mixed with primary antibodies in PBSB for 90 min, followed by incubation with fluorescent dye-conjugated secondary antibodies for 30 min. For cholesterol staining we used the fluorescent polyene antibiotic filipin (Sigma), which interacts with free cholesterol at the 3-$\beta$-hydroxyl group (Mauch et al., 2001). After fixation the cells were stained with 50 $\mu$g/ml filipin for 30 min. Fluorescence images were obtained via a laser confocal microscope (RTS2000; Bio-Rad, Hercules, CA) or with a cooled CCD camera (MRc5; Carl Zeiss, Thornwood, NY) mounted on an epifluorescence microscope (TE230; Nikon, Tokyo, Japan). Captured images were processed via Adobe Photoshop (see Figs. 1A, 3, 6A). For quantitative analysis the numbers of synaptophysin-positive puncta were determined by using Scion (Frederick, MD) Image software (Suzuki et al., 2004). First we set the threshold for detecting fluorescent spots above the background intensity of that cell and normalized the intensity of the fluorescent spots to the background intensity. The number was estimated as a total area of fluorescent spots and normalized to that of MAP-2-positive cells.

*Real-time reverse transcriptase-PCR.* Cortical neurons or glial cells were treated with BDNF for the indicated times after overnight starvation, using Neurobasal medium without B27 supplements. Total RNA was isolated from cultured neurons by using the ISOGEN reagent (Nippon Gene, Toyama, Japan), following the manufacturer's instructions. cDNA synthesis and quantitative detection were performed with a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA), using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The primers for each gene were designed on the basis of the rat sequences: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer 5′-TGCAC CACCA ACTGC TTAG-3′ and reverse primer 5′-GGATG CAGGG ATGAT GTTC-3′), HMG-CoA reductase (forward primer 5′-GATCT GTGGT TGGAA TTATG AGTGC-3′ and reverse primer 5′-TGTAC TTTGA CCCAA GCTGA CG-3′), mevalonate pyrophosphate decarboxylase (forward primer 5′-GGATG AAGCG CTGAT CCTG-3′ and reverse primer 5′-GGTCC TCTGT GAAGT CCTTG-3′), squalene synthase (forward primer 5′-CGGAA TTTCA TACCC AAGAT GGAC-3′ and reverse primer 5′-CGAAG CTGCG ACTGG TCTG-3′), and sterol regulatory element-binding protein-2 (SREBP-2) (forward primer 5′-GAGAC CATGG AGACC CTCAC-3′ and reverse primer 5′-GAGAA CTCTC CCACT TGATT GCTG-3′). To confirm the amplification specificity, we subjected PCR products to dissociation curve analysis and gel electrophoresis on a 2% agarose gel to ensure the melting temperature and size of the DNA fragment. Expression levels for each mRNA were estimated by normalization to GAPDH levels measured in the same samples.

*Cell viability assay.* Cell viability was estimated by diamidinophenylindole (DAPI) staining, which also enabled us to count the number of apoptotic cells, which have condensed nuclei, and the number of normal, intact cells. Cell types were determined by staining with antibodies to MAP-2, GFAP, or O4 to identify neurons, astrocytes, and oligodendrocytes, respectively.

*Electrophysiology.* For whole-cell recordings pyramidally shaped neurons were selected visually by using DIC optics and continuous flow (1–2 ml/min) of HEPES-buffered artificial CSF (aCSF-H) containing the following (in mM): 124 NaCl, 5 KCl, 30 glucose, 2 $CaCl_2$, 1 $MgCl_2$, 0.05 picrotoxin (Takeda Chemical Industries, Yokohama, Japan), and 25 HEPES, pH 7.4 (Dojin, Tokyo, Japan), plus 0.5 $\mu$M tetrodotoxin. Solutions in patch pipettes contained the following (in mM): 110 Cs-methanesulfonate, 10 $CsCl_2$, 20 HEPES, 4 $MgCl_2$, 2.5 EGTA-Cs, 0.3 GTP-$Na_3$, and 4 ATP-$Na_2$, pH 7.3. Miniature EPSCs (mEPSCs) were recorded with a −70 mV holding potential for 5 min with the MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA) and controlled by a pClamp9 device (Molecular Devices). mEPSCs (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) were analyzed by Clampfit or Mini Analysis (Synaptosoft, Decatur, GA). For the analysis of neurotransmitter release caused by osmotic shock, sucrose was applied with a glass pipette for whole-cell recordings on primary dendrites near the cell body (see Fig. 7) (Rosenmund and Stevens, 1996) with a picopump (World Precision Instruments, Sarasota, FL). For osmotic shock 100 mM sucrose-containing aCSF-H was applied for 35 s, and the recording was started 5 s after this stimulation. Sucrose-evoked EPSCs (see Fig. 7) were analyzed by Clampfit or Mini Analysis (Synaptosoft). All chemicals for electrophysiological experiments were obtained from Wako, unless otherwise specified.

*Statistical analysis.* Data are presented as the means ± SEM. Student's *t* test and one-way ANOVA, together with Bonferroni as a *post hoc* test, were used for statistical analysis.

## Results

### BDNF increases cholesterol content in cultured cortical neurons

We first examined whether BDNF controlled the cholesterol content of neurons by measuring the cholesterol contents of cortical neurons cultured in the presence or absence of BDNF. The soma and dendrites of the cortical neurons were stained by the cholesterol marker filipin (Fig. 1A). The cholesterol contents were quantified by using cholesterol oxidase in an enzyme-coupled colorimetric assay (see Materials and Methods) (Suzuki et al., 2004). In the present study the neurons were cultured by using serum-free Neurobasal medium and contained the B27 supplement and glutamine when indicated (from 3–4 to 6–7 DIV). This medium does not contain cholesterol or any precursor used for cholesterol biosynthesis (Brewer et al., 1993) and, as described below, suppresses the prosurvival effect of BDNF on cultured neurons.

We found that a 3 d treatment with BDNF (200 ng/ml) increased the level of cholesterol in cultured cortical neurons by 38 ± 1% (Fig. 1B) (control, 116 ± 2 ng/ml; BDNF, 160 ± 2 ng/ml; $p < 0.01$; $n = 3$ independent cultures). Cultured hippocampal neurons also increased the cholesterol content in response to BDNF (Fig. 1B) (control, 96 ± 2 ng/ml; BDNF, 115 ± 3 ng/ml; $p < 0.01$; $n = 3$ independent cultures). As well as increasing tyrosine phosphorylation of the TrkB receptor, the cholesterol content was increased significantly by BDNF in a dose-dependent manner (Fig. 1C) ($p < 0.03$). In control cultures activation of the TrkB receptor was not detected (Fig. 1C, top), and endogenous BDNF in the medium was below the detection limit (data not shown), suggesting that endogenous BDNF is insufficient to raise the cholesterol content of the culture. In a time course study a significant increase of cholesterol was observed at 3 d after BDNF application (Fig. 1D). To verify that the effect of BDNF was specific for cholesterol biosynthesis, we extracted sterols from cultured cortical neurons and separated them via TLC (Metherall et al., 1996). The major sterol bands on the TLC plates were cholesterol and lanosterol. BDNF stimulation (200 ng/ml; 5 d) appeared to raise the intensity of cholesterol band specifically (Fig. 1E, black arrowhead), and densitometric