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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No association between the glutamate decarboxylase 67 gene (GAD1) and schizophrenia in the Japanese population

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Abstract

Postmortem studies regarding schizophrenia revealed altered expression of genes related to γ-amino butyric acid neurotransmission system. One of the most consistent findings is the reduced level of 67 kDa glutamic acid decarboxylase isoform (GAD_{67}). Moreover, several studies reported positive associations between the GAD_{67} gene (GAD1) and schizophrenia. These reasons, motivated us to carry out replication study regarding association between GAD1 (fourteen tagging SNPs) and schizophrenia in Japanese population (562 schizophrenic patients and 470 controls). However we couldn't confirm significant association that had been previously reported. Considering size of our sample and strategy that corresponds well with the approaches used in gene-based association analysis, our conclusion is that GAD1 does not play a major role in schizophrenia in Japanese population.

Keywords: Schizophrenia; GAD1; Linkage disequilibrium; GABA; Association study

1. Introduction

Abnormalities in the γ-amino butyric acid (GABA) neurotransmission system are thought to be involved in the pathophysiology of schizophrenia. Several postmortem studies showed altered expression of genes related to GABA in schizophrenic patients. One of the most consistent findings is related to reduced level of 67 kDa glutamic acid decarboxylase isoform (GAD_{67}), key enzyme in GABA synthesis (Lewis et al., 2005).

The GAD_{67} is encoded by the glutamic acid decarboxylase 1 gene (GAD1) located on 2q31, and two studies reported positive associations between GAD1 and schizophrenia (Straub et al., 2003; Addington et al., 2005). Interestingly, those studies showed positive association of: 1) childhood onset schizophrenia (COS) and 2) cortical gray matter volume loss with schizophrenia (Addington et al., 2005). Moreover, a

Abbreviations: γ-amino butyric acid, (GABA); 67 kDa isoform of glutamic acid decarboxylase, (GAD_{67}); Glutamic acid decarboxylase 1 gene, (GAD1); Childhood onset schizophrenia, (COS); Adult onset schizophrenia, (AOS); Single nucleotide polymorphism, (SNP); Linkage disequilibrium, (LD); Minor allele frequency, (MAF); Genotype relative risk, (GRR); Age at onset, (AAO).

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significant association between GAD1 and schizophrenia in two independent adult onset schizophrenia (AOS) samples has been reported as well, suggesting biological continuity between COS and AOS (Straub et al., 2003).

In contrast, three other studies reported no association between GAD1 and schizophrenia, although, due to sample size, possibility of type II error cannot be excluded (De Luca et al., 2004; Lundorf et al., 2005; Zhang et al., 2005).

It is widely accepted that there are limitations in interpreting the results of simple replication studies that examine the same or a smaller number of single nucleotide polymorphisms (SNPs) as in the original study. Aforementioned can be explained by the differences in allele frequency or variation of linkage disequilibrium (LD) structure (population dependence). To overcome these limitations, a gene-based approach rather than an SNP-based or haplotype-based approach is currently recommended (Neale and Sham, 2004). In such studies it is important to: (1) include both gene and gene flanking regions in testing for association, and (2) select genetic variants which adequately reflect the LD background in the targeted population (e.g. tagging SNPs).

Applying this gene-based association concept, we tested the association between GAD1 tagging SNPs and schizophrenia using relatively large samples in the Japanese population.

2. Materials and methods

2.1. Subjects

The sample used in this study was comprised of 562 schizophrenia patients (301 males and 261 females; range=15–82 years old, median=44 years old, mean±SD=44.9±15.2 years) and 470 healthy controls (269 males and 201 females; range=19–95 years old, median=34 years old, mean±SD=37.5±14.6 years). All subjects were unrelated and with Japanese ethnicity. Subject attributes and psychiatric assessment were identical to those described elsewhere (Ikeda et al., 2005).

Subsequent to study description, written informed consent was asked from each subject. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University.

2.2. SNP selection

We first included marginal and significant SNPs (Markers 4, 5, and 6) reported by others (Addington et al., 2005) and a potent functional SNP (Marker 3; located 2246 base pairs (bp) upstream from the initial exon and Marker 12; located in 3′UTR) (Table 1). Next, we consulted the HapMap database (release#21, population: Japanese in Tokyo (JPT), minor allele frequency (MAF): more than 0.01). In this step, we determined the boundaries of the GAD1 gene that covered 5′-flanking regions including 5000 bp from the initial exon and 1700 bp downstream (3′) from the last exon (GenBank accession No. NM_000817: Supplementary Figure 1). Then eleven 'tagging SNPs' were selected with the criterion of an r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (de Bakker et al., 2005), implement of HAPLOVIEW software (Barrett et al., 2005). These tagging SNPs were used in the subsequent association analysis (since Markers 4 and 6 were listed in the HapMap data, they were force-included with these 'tagging SNPs'). Overall, 14 tagging SNPs were examined.

2.3. SNP genotyping

All SNPs were genotyped by TaqMan assay (Applied Biosystems Japan Ltd, Tokyo). Detailed information, including reaction conditions, can be seen in another paper (Ikeda et al., 2005).

2.4. Statistical analysis

Marker-trait association was evaluated by the χ^2 test (allele and genotype-wise analyses). For haplotype-wise analysis, LD blocks were initially defined in accordance with Gabriel's criteria, and haplotype frequencies were estimated in each LD block with the Expectation–Maximization algorithm. Log likelihood ratio tests were performed for global *P*-values (COCAPHASE 2.403 program, Dudbridge, 2003). Power calculation was performed with a web-based statistical program, Genetic Power Calculator (Purcell et al., 2003). Power was estimated under multiplicative model of inheritance, assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values in observed in control samples.

3. Results

All genotype frequencies of these SNPs were in Hardy–Weinberg equilibrium. The LD matrices of the 14 tagging SNPs we tested are shown in Supplementary Figure 2. No association was found between cases and controls in allele-, genotype- or haplotype-wise analyses (Table 1).

Table 1
Association analysis of tagging SNPs in GAD1

SNP ID[a]		Positions[b]	Blocks[c]	*N*		Genotype distribution[d]						MAFs[e]			*P*-value		
						M/M		M/m		m/m							
				SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON		Genotype	Allele	Haplotype
Marker 1	rs1978340	1	I	561	466	371	306	175	148	15	12	.18	.18	(.31)	.978	.915	.549
Marker 2	rs3762561	625	I	561	469	368	292	165	158	28	19	.20	.21	(0)	.297	.500	
Marker 3	rs12185692	704		561	466	269	237	244	194	48	35	.30	.28	(.37)	.610	.328	
Marker 4	rs3749034*	3353	II	561	466	244	211	265	216	52	39	.33	.32	(.16)	.795	.517	.194
Marker 5	rs2270335*	4574	II	561	466	245	212	265	214	51	40	.33	.32	(0)	.836	.574	
Marker 6	rs2241165*	8257	II	561	466	244	212	265	215	52	39	.33	.31	(.19)	.768	.484	
Marker 7	rs3828275	12618		561	467	266	224	247	200	48	43	.31	.31	(.49)	.894	.980	
Marker 8	rs2241164	17437		562	470	189	142	271	243	102	85	.43	.44	(.31)	.459	.444	
Marker 9	rs769407	23586	III	561	468	314	261	215	179	32	28	.25	.25	(.24)	982	.900	438
Marker10	rs3791851	28552	III	561	467	298	238	226	191	37	38	.27	.29	(.25)	.582	.350	
Marker 11	rs3791850	37978	III	561	468	431	370	119	93	11	5	.13	.11	(.18)	.429	.275	
Marker 12	rs769395	46681	III	561	466	287	233	237	193	37	40	.28	.29	(.27)	.484	.431	
Marker 13	rs16858996	48980		561	468	370	295	176	153	15	20	.18	.21	(.11)	.302	.197	
Marker 14	rs17701824	49124		561	468	266	221	231	204	64	43	.32	.31	(.43)	.458	.622	

Numbers in parenthesis represent MAFs in Caucasians (cited from HapMap database and Applied Biosystems database).
[a] IDs with asterisk represent significant or marginally significant SNPs in Addington's report.
[b] Based on Accession No. NT005403.16.
[c] Determined by HAPLOVIEW.
[d] *N* = number, M = major allele, m = minor allele, SCZ = schizophrenia, CON = control.
[e] MAFs = minor allele frequencies.

Power analyses showed that the power was more than 80% when genotype relative risk (GRR) was set at 1.3–1.5 under a multiplicative model of inheritance.

4. Discussion

In this study, we were unable to confirm an association between GAD1 and schizophrenia in the Japanese population.

Addington et al. (2005), using an *in silico* approach, reported that positive SNPs in the 5′ region (Marker 4 and 5 in our study) of GAD1 possibly have various effects on gene expression. They speculated that these SNPs may alter the expression level of GAD1. Their findings supported previous postmortem studies showing down-regulation of GAD67 in schizophrenics. This data suggests that the 5′-flanking region of this gene might harbor schizophrenia-susceptibility factor. However, our results do not support the positive association (Markers 4 and 5) in the 5′-flanking region of GAD1 previously reported by Addington, even though we examined the other three SNPs in the 5′-flanking region (Markers 1, 2 and 3) in addition to Addington's two SNPs. Our sample size also showed quite high power. From the viewpoint of the common disease-common variant hypothesis (Chakravarti, 1999), our data didn't provide the evidence that GAD1 have a major role in schizophrenia in the Japanese population.

We also included an explorative analysis of gender effect and age at onset (AAO: $N=310$), for the following reasons: 1) Straub et al. (2003) and Addington et al. (2005) reported that relations were significantly greater or stronger in females, and 2) this analysis allowed us to consider the relation between the negative results of our AOS samples in Japanese and the positive COS samples in Caucasians. However, no associations were found in analysis subdivided by gender, or between AAO and GAD1 allele and haplotypes (evaluated by haplotype trend regression analysis; Liu and Muse, 2005: data not shown).

Although the strategy we used for the present association analysis corresponded well with that used in gene-based association analysis (Neale and Sham, 2004), several limitations must be outlined. First, in case of examination of possible association between GAD1 and schizophrenia, causal variants with extremely rare MAFs, allelic heterogeneity, or locus heterogeneity should be considered (Neale and Sham, 2004). In such situations, quite large sample sizes are needed for rare variants searching; i.e. more than 3500 cases and controls are required for 80% power when GRR is set at 1.5 and MAF is 1% (Purcell et al., 2003). Second, our control subjects were significantly younger than case subjects. Thus, our control samples may have included a number of individuals below the age of peak risk for schizophrenia-onset, and this confounding factor has potential for decreasing power of this study. Third, GAD1 may influence the function and morphology of the dorsolateral prefrontal cortex (Addington et al., 2005; Lewis et al., 2005). Therefore, endophenotypic approaches such as cognitive function, brain imaging and other phenotypes reflecting characteristics of GAD1 will be needed in the future (Gottesman and Gould, 2003).

In conclusion, our gene-based analysis of GAD1 showed no association between this gene and AOS in the Japanese population. Further studies using different population samples will be needed to conclude whether GAD1 is a race specific or rare phenotype specific susceptibility factor for AOS.

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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.2006.12.020.

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Behavioral/Systems/Cognitive

A Cyclooxygenase-2 Inhibitor Ameliorates Behavioral Impairments Induced by Striatal Administration of Epidermal Growth Factor

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Consistent with the hypothesis that neuroinflammatory processes contribute to the neuropathology of schizophrenia, the protein levels of epidermal growth factor (EGF) and its receptor ErbB1 are abnormal in patients with schizophrenia. To evaluate neuropathological significance of this abnormality, we established an animal model for behavioral deficits by administering EGF into the striatum and evaluated the effects of cyclooxygenase-2 (Cox-2) inhibitor celecoxib. Intracranial infusion of EGF into the striatum of adult male rats activated ErbB1 and induced neurobehavioral impairments observed in several schizophrenia models. Unilateral EGF infusion to the striatum lowered prepulse inhibition (PPI) in a dose-dependent manner and impaired latent learning of active shock avoidance without affecting basal learning ability. Bilateral EGF infusion similarly affected PPI. In contrast, EGF infusion to the nucleus accumbens did not induce a behavioral deficit. Intrastriatal EGF infusion also increased Cox-2 expression, elevated tyrosine hydroxylase activity, and upregulated the levels of dopamine and its metabolites. Subchronic administration of celecoxib (10 mg/kg, p.o.) ameliorated the abnormalities in PPI and latent learning as well as normalized dopamine metabolism. We conclude that this EGF-triggered neuroinflammatory process is mediated in part by Cox-2 activity and perturbs dopamine metabolism to generate neurobehavioral abnormalities.

Key words: **inflammation; cyclooxygenase; EGF; schizophrenia; dopamine; prostaglandin**

Introduction

In the CNS, epidermal growth factor (EGF) and structurally related EGF-like peptides (ErbB1 ligands) enhance survival and neurite outgrowth of midbrain dopaminergic neurons and are implicated in dopamine (DA)-related brain diseases such as Parkinson's disease and schizophrenia (Casper et al., 1994; Farkas and Krieglstein, 2002). EGF content is decreased and EGF receptor (ErbB1) levels are increased in the striatum of schizophrenia patients (Futamura et al., 2002). Genetic linkage studies may also support the contribution of EGF to schizophrenia etiology or pathology (Anttila et al., 2004; Hanninen et al., 2007). EGF and other EGF homologs were isolated as cell growth factors and implicated in the progression of cancer (Ackerman et al., 2004; Slice et al., 2005; Liao et al., 2006). Binding of EGF to ErbB1 enhances the expression of inducible prostaglandin synthetase [cyclooxygenase 2 (Cox-2)] and triggers a variety of inflammatory processes (Slice et al., 2005; Liao et al., 2006). Therefore, EGF and other homologs are implicated in the pathogenesis of inflammatory diseases such as rheumatoid arthritis. Subsequently produced prostaglandins bind G-protein-coupled receptors and stimulate production of EGF or other ErbB1 ligands by ectodomain shedding (Pai et al., 2002; Han et al., 2006). This feedforward loop between ErbB1 ligands and Cox-2 expression promotes cancer cell proliferation and inflammatory progression (Huh et al. 2003). In contrast, the central actions of EGF or other ErbB1 ligands on the neuroinflammatory processes are poorly understood.

Neuroinflammation is implicated in etiology or neuropathology of not only neurodegenerative diseases but also a number of psychiatric disorders (Das and Khan, 1998; Heleniak and O'Desky, 1999). Patients with schizophrenia often exhibit impaired autoimmune responses and abnormal levels of cytokines (Licinio et al., 1993; Lin et al., 1998; Toyooka et al., 2003). Interestingly, there is also a reverse correlation between neuroinflammation and psychiatric symptoms. Various psychiatric symptoms develop during or after cytokine therapy for cancer, viral infection, and anemia (Denicoff et al., 1987; McDonald et al., 1987). Thus, cytokine-mediated inflammatory reactions may in certain circumstances evoke psychiatric symptoms. Based on this hypothesis, preclinical trials of nonsteroidal anti-inflammatory drugs on patients with schizophrenia are underway (Muller et al., 2002, 2004; Riedel et al., 2005). Tetracycline, an antibiotic that possesses anti-inflammatory activity, improves the positive and negative syndrome scale (PANSS) of schizophrenia patients (Miyaoka et al., 2007). Thus, anti-inflammatory medication aug-

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ments therapies for neuropsychiatric conditions, although the pharmacological mechanisms underlying their antipsychotic actions are not fully characterized.

To study a potential neuroinflammatory role of the striatal EGF signal in a schizophrenia model, we examined the neurobehavioral consequences of striatal EGF administration as well as its effects on Cox-2 expression in rats. Given the biological activity of EGF on dopaminergic neurons, we focused on the neurochemical markers and animal behaviors related to dopaminergic dysfunctions and/or schizophrenia, prepulse inhibition (PPI) of startle, and latent inhibition (Braff et al., 2001; Geyer et al., 2001; Jeanblanc et al., 2003; Peterschmitt et al., 2005). We also evaluated the effects of acute and subchronic inhibition of Cox-2 in conjunction with dopamine metabolism and behavioral performance.

Materials and Methods

Subjects. Male Sprague Dawley rats (7–8 weeks old, 300–380 g body weight, 190 rats in total) were purchased from SLC (Shizuoka, Japan) and maintained under a 12 h light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.) with access to food and water *ad libitum*. Before testing, rats were habituated to experimental rooms and experimenters with daily handling for at least 1 week. Surgical operation and behavioral tests were performed during the day cycle. Recombinant human EGF (Higeta Syoyu, Chiba, Japan) was dissolved in saline and intracranially administered from an osmotic minipump (see below). All of the animal experiments described here were performed in accordance with the Animal Use and Care Committee guidelines of Niigata University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Surgical procedure and subchronic EGF administration. Rats (8–9 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Dainippon Pharmaceutical, Suita, Osaka, Japan). After confirming the deep anesthesia, each rat was mounted on a stereotaxic apparatus (Narishige, Tokyo, Japan) with the upper incisor bar set 3.0 mm below the interaural line. The skull was exposed and a hole drilled for unilateral placement of intracerebral cannula guides into either the striatum or nucleus accumbens. A cannula (30 gauge; Terumo, Tokyo, Japan) was implanted in the striatum (0.5 mm posterior and 3.0 mm right lateral measured from the bregma, 5.5 mm below the skull) or in the nucleus accumbens (1.6 mm anterior and 2.0 mm right lateral measured from the bregma, 7.0 mm below the skull), glued to the skull, and connected to an Alzet osmotic minipump (model 2002, a 2 week type; Alza, Palo Alto, CA) by medical-grade vinyl tubing. For bilateral EGF infusion, cannulas were implanted in both hemispheres of the striatum (0.5 mm posterior and ±3.0 mm lateral measured from the bregma, 5.5 mm below the skull) and connected to two minipumps. A minipump was filled with human recombinant EGF (0.005, 0.05, or 0.15 mg/ml; 0.2 ml total) or 0.9% saline (vehicle) and implanted subcutaneously in the nape of the neck. Either saline or EGF was administered continuously at a rate of 0.5 μl/h from the minipump. Unless the dose of EGF is otherwise specified, each minipump contained 30 μg of protein and EGF was infused at a rate of 75 ng/h. The scalp incision was closed with surgical staples and treated with a topical antiseptic, Cefmetazon (50 mg/d; Sankyo Pharmaceuticals, Tokyo, Japan). The cannula position and EGF content in a pump were confirmed after completion of behavioral tests.

Schedule of behavioral testing, drug treatment, and dissection. Rats were subjected to behavioral tests after a recovery period at least 7 d in length after minipump implantation but before the minipump was depleted of EGF (14 d after surgery). The saline- or EGF-infused rats were additionally treated with the Cox-2 inhibitor celecoxib. Celecoxib (10 mg/kg; Pfizer, New York, NY) was dissolved in saline and administered once a day orally with the aid of oral zonde for rats (Natusme Seisakusho, Tokyo, Japan). The celecoxib treatment was performed daily 3–4 d after pump implantation to days 11–12 after surgery. Behavioral tests followed this treatment regimen. To minimize the acute influences of celecoxib, examinations were performed at least 20 h after treatment unless it is otherwise specified.

To avoid interactions between independent behavioral tests, rats were subjected to one of the scheduled tests only once. The sole exception to this testing strategy was that rats that had been subjected to PPI testing were also tested for context learning. After behavioral evaluation, most of rats were killed, and tissue was harvested for neurochemical analysis or histochemistry (see below). Tissue samples for basal monoamine turnover were harvested from rats examined for effects of EGF doses on PPI. The effects of celecoxib on monoamine turnover were examined with tissue from animals tested for latent learning. The rats receiving EGF in the nucleus accumbens were fixed for histochemistry after PPI testing. To minimize the influences of the behavioral tests on the levels of monoamines, their extraction was performed at least 6 h after the end of the test. All other tissue samples were prepared from the rats that did not receive behavioral testing. Animals that exhibited any symptoms of infection around the cannula or the incision site were removed from study. In total, eight experimental groups representing 180 rats were analyzed in the present study.

Histochemistry. Rats receiving EGF infusion were transcardially perfused with 4% paraformaldehyde in a 0.1 M PBS, pH 7.4. Coronal sections (15 μm thick) were cut from frozen brains and immunostained with antibodies directed against human EGF (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or Cox-2 (1:500; Cayman Chemical Ann Arbor, MI). The immunoreactivity was visualized with biotinylated anti-rabbit Ig antibody coupled with the ABC method (Vector Laboratories, Burlingame, CA).

Immunoblot analysis. Rat receiving EGF or saline were killed by carbon dioxide exposure, and the brains were dissected out. Samples of striatum were homogenized by ultrasonication in 2× sample loading buffer (100 mM Tris-HCl buffer, pH 6.8, 4% SDS, 100 mM dithiothreitol, 20% glycerol, and 0.0001% bromophenol blue). Protein extracts (5 or 50 μg per lane) were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membranes by electrophoresis. Membranes were probed with antibodies directed against ErbB1 (1:1000; Santa Cruz Biotechnology). Alternatively, immunoblots were probed with anti-phosphorylated ErbB1(1:1000), anti-ErbB2 (1:2000), anti-D_2 receptor (1:1000), anti-dopamine transporter (DAT) (1:1000), anti-Cox-2 (1:1000; all from Santa Cruz Biotechnology), anti-D_1 receptor (1:1000; Sigma, St. Louis, MO), anti-synaptophysin (1:1000; NeoMarkers, Fremont, CA), or anti-tyrosine hydroxylase (1:1000; Chemicon, Temecula, CA) antibodies. The immunoreactivity on the membrane was detected by chemiluminescence (ECL kit; GE Healthcare, Little Chalfont, UK).

The activity of tyrosine hydroxylase. Striatal tissue was homogenized in lysis buffer [0.32 M sucrose, 20 mM Tris-HCl, pH 7.3, 1 mM dithiothreitol, and protease inhibitor cocktail (Complete Mini; Roche Diagnostic, Mannheim, Germany)]. Protein homogenates (20 μl/tube) were incubated with 180 μl of reaction buffer [0.2 mM L-tyrosine, 0.2 M sodium acetate, 0.1 M 2-mercaptoethanol, 0.5 mM ferrous sulfate heptahydrate, 1 mM 6-methyl-5,6,7,8-tetrahydropterin (Sigma), 0.2 mg/ml catalase (Roche Diagnostic), and 50 μM S(−)-carbidopa (Wako Chemical, Tokyo, Japan)] at 37°C for 10 min. The enzyme reaction was terminated on ice with a stop solution (250 μl) [1 M perchloric acid, 0.2 M EDTA, and 1 mM α-methyldopa (an internal standard)]. After 15 min incubation on ice, 150 μl of 1 M K_2CO_3 and 1 ml of 0.1 M Tris-HCl, pH 8.5, were added, and the supernatant was collected. The product of 3,4-dihydroxy-L-phenylalanine (L-DOPA) was absorbed with alumina (aluminumoxide 90 active acidic; Merck, Darmstadt, Germany) and then eluted with 0.5 M HCl (0.8 ml/tube). Concentrations of L-DOPA were determined by HPLC with electrochemical detection ECD (see below) (mobile phase: 50 mM trisodium citrate, 25 mM NaH_2PO_4, 100 μM EDTA, and 1% methanol, pH 2.8).

Measurement of acoustic startle and prepulse inhibition. Acoustic startle and PPI responses were measured in a startle chamber (SR-Lab Systems; San Diego Instruments, San Diego, CA) adapted for rats (Braff and Geyer, 1990; Swerdlow and Geyer 1998; Swerdlow et al., 2001). The chosen paradigm was adapted and modified from Braff and Geyer (1990) and used to assess startle amplitude, habituation, and PPI response with acoustic stimuli of 120 dB, a single prepulse interval (100 ms), and three

Table 1. Effects of striatal EGF infusion on monoamine contents and turnover

	Saline (0 μg)	3 μg of EGF	10 μg of EGF	30 μg of EGF
5-HIAA	326 ± 69	428 ± 105	480 ± 62	498 ± 103
5-HT	17,200 ± 4200	25,800 ± 7610	24,600 ± 2250	20,200 ± 3320
DA	109,000 ± 2690	109,000 ± 37,900	157,000 ± 12,300	184,000 ± 5780*
DOPAC	1220 ± 213	1230 ± 373	1850 ± 152	2020 ± 152*
HVA	2400 ± 580	3440 ± 1140	5580 ± 423**	5030 ± 502**
EP	47 ± 5	43 ± 4	47 ± 3	47 ± 3
NE	404 ± 62	590 ± 159	394 ± 54	490 ± 35

Different doses of EGF were unilaterally administered to rat striatum from a minipump for 10 d, and monoamines were extracted from the ipsilateral hemisphere of the striatum. The levels of DA, DOPAC, HVA, serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in the striatum were determined by HPLC-ECD as described previously (Futamura et al., 2003). The total amounts of EGF in a minipump were given in this table. Data represent means ± SEM (picomoles per gram of wet tissue; $n = 5$–6 animals each). *$p < 0.05$, **$p < 0.01$ by Fisher's LSD. EP, Epinephrine; NE, norepinephrine.

different prepulse intensities [5, 10, and 15 dB above background noise (white noise, 70 dB)]. Each rat was placed in the startle chamber and initially acclimatized for 5 min with background noise alone. The rat was then subjected to 50 startle trials, each trial consisting of one of five conditions: (1) a 40 ms, 120 dB noise burst presented alone; (2–4) a 40 ms, 120 dB noise burst after prepulses by 100 ms (20 ms noise burst) that were 5, 10, or 15 dB above background noise (i.e., 75, 80, or 85 dB prepulse, respectively); or (5) no stimulus (background noise alone), which was used to measure baseline movement in the chamber. In PPI tests, these five trial types (1–5) were each repeated eight times in a pseudorandom order to give 40 trials. Each trial type was presented once within a block of five trials. At the beginning and end of the PPI test, five consecutive trials of condition 1 were presented to assess habituation during sessions. The intertrial interval was 15 s. Analysis of PPI was based on the mean of the eight trials for each trial type. The percentage PPI of a startle response was calculated as follows:

$$\mathrm{PPI} = 100 - \frac{(\text{prepulse and pulse stimulus trials} - \text{no stimulus trials}) \times 100}{\text{pulse-alone trials} - \text{no stimulus trials}}.$$

Active-avoidance learning and latent inhibition. Rats were given 60 trials of two-way active-avoidance conditioning (10 trials per block) (Salmi et al., 1994; Futamura et al., 2003). Active-avoidance testing was conducted in an automated shuttle box (Muromachi-kiki, Tokyo, Japan) subdivided into two virtual compartments with independently electrified stainless-steel bars as floors. One trial consisted of a buzzer tone [conditioning stimulus (CS)] and an electric shock [unconditioning stimulus (US)]. The CS was an 80 dB tone for 10 s. The US was a 2 s positive half-wave constant current of 0.5 mA intensity. When the CS was on, the animals had to cross to the other side of the shuttle box apparatus (avoidance response) to turn the CS off and to avoid the US. The US was initiated if the animal failed to make an escape response. The intertrial schedule had a variable interval (10–90 s). Animals in non-preexposed (NPE) group were directly subjected to the above conditioning. One day before the conditioning, rats in the preexposed (PE) group were placed in the automated shuttle box and exposed to the buzzer tones (CS) with the above protocol but without receiving electric stimuli (US). Preexposed to CS were followed by the final treatment with celecoxib at an interval of 1 h.

Context fear learning. The test paradigm of contextual conditioning was based on a work by Matus-Amat et al. (2004). Rats were transported to the laboratory at least 30 min before fear conditioning. Rats were placed in a shock chamber with a stainless-steel grid floor (21.5 cm width × 20.5 cm depth × 30 cm height box; Ohara Medical Industry, Tokyo, Japan) for 2 min to monitor baseline movement/freezing and were then exposed to 0.8 mA electric shocks (2 s duration, twice at an interval of 1 min). One day after conditioning, rats were returned to the same chamber. The time spent freezing was recorded by a video camera and averaged every 30 s with the aid of an imaging software (Ohara Medical Industry). The final treatment with celecoxib was done 1 d before conditioning.

Quantification of dopamine and its metabolites. The levels of DA, L-DOPA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin, 5-hydroxyindoleacetic acid, epinephrine, and norepinephrine were determined by HPLC-ECD as described previously (Futamura et al., 2003). Frontal cortex and striatum were dissected out from rats, weighed, and then immediately homogenized by ultrasonication in 0.5 ml of 0.1 M perchrolic acid containing 0.1 mM EDTA and 250 nM isoproterenol as an internal standard. The homogenate was placed on ice for 30 min and then centrifuged at 10,000 × *g* for 10 min. The HPLC-ECD system consisted of a pump (model LC-10ADVP; Shimadzu, Kyoto, Japan), an automatic sample injector (model SIL-10ADVP; Shimadzu), a C18 column (model CA-5ODS, 4.6 × 150 mm; Eicom, Kyoto, Japan), and an electrochemical detector with a glassy carbon-working electrode (model ECD-300; Eicom). The mobile phase consisted of 50 mM trisodium citrate, 25 mM NaH_2PO_4, 0.03 mM EDTA, 10 mM diethylamine, 3 mM octanesulfonic acid sodium salt, 6% methanol, and 1% dimethylacetamide, pH 3.2. The current produced was monitored using an EPC-300 (Eicom).

Statistical analysis. Results were expressed as means ± SEM. Statistical differences were determined by ANOVA. When univariate data were obtained only from two groups, a two-tailed *t* test was used for comparison. Behavioral scores were initially analyzed using multiple ANOVA with EGF administration (two levels or four doses), celecoxib treatment (two levels), and/or preexposure to CS (two levels) as between-subject factors and prepulse magnitude (three levels) or block (six) as a within-subject factor. Interaction of a within-subject factor with between-subject factors was estimated by analysis of covariance and multivariate analysis of variance with Pillai compensation. Because the initial analyses yielded significant factorial interaction, the data were separated to avoid the interaction for the final analyses. Subsequently, a Fisher's least significant difference (LSD) test was applied to absolute behavioral values as a *post hoc* test of multiple comparisons. A p value <0.05 was regarded as statistically significant. Statistical analysis was performed using Statview software (SAS Institute, Cary, NC). n values in parentheses represent the number of animals used.

Results

Effects of intrastriatal EGF administration on monoamine metabolism and sensorimotor gating

Peptides in the EGF family exert neurotrophic effects on dopaminergic neurons both *in vitro* and *in vivo* (Casper et al., 1994; Farkas et al., 2002; Futamura et al., 2003; Iwakura et al., 2005) and increase the activity of tyrosine hydroxylase (Halegoua and Patrick, 1980; Anastasiadis et al., 1997). To investigate the central actions of EGF, we unilaterally administered various concentrations of EGF to the striatum of rats with an osmotic minipump and then determined the levels of monoamines and their metabolites in brain tissue. Subchronic EGF infusion to the striatum increased the levels of dopamine ($F_{(3,17)} = 3.48$; $p = 0.039$) and its metabolites DOPAC and HVA ($F_{(3,17)} = 4.03$, $p = 0.025$ for DOPAC and $F_{(3,17)} = 5.27$, $p = 0.009$ for HVA) in the striatum in a dose-dependent manner (Table 1). *Post hoc* comparisons revealed that only the highest dose of EGF (30 μg/pump, 1.8 μg/d) significantly increased the levels of dopamine, DOPAC, and HVA. In contrast to the effects on the striatum, EGF did not alter

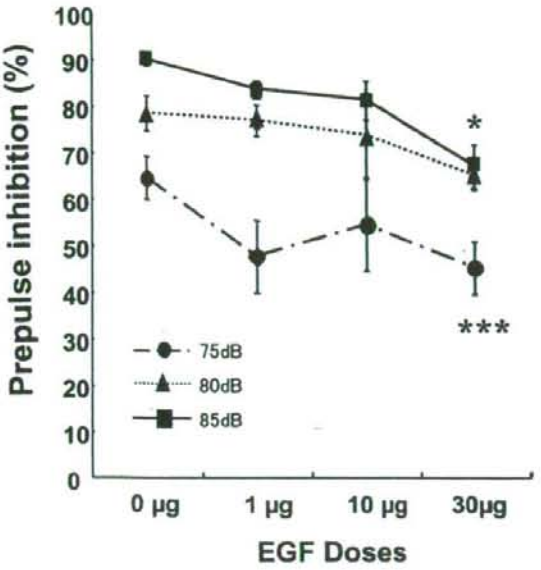

Figure 1. EGF dose dependency of PPI deficits. Different doses of human EGF (0, 1, 10, and 30 μg/pump, equivalent to 0, 0.06, 0.6, and 1.8 μg/d) were administered to the striatum of male adult rats from an osmotic minipump for 10 d. PPI with 75, 80, and 85 dB prepulse stimuli was measured and compared between doses. Values indicate means ± SEM (n = 7–10 each). $^{*}p < 0.05$, $^{***}p < 0.001$ compared with vehicle-infused controls by Fisher's LSD.

the concentrations of dopamine and its metabolites in frontal cortex (data not shown).

To evaluate the neurobehavioral consequences of intrastriatal EGF infusion, we also monitored the dose-dependent effects of EGF on PPI (Fig. 1). A two-way repeated ANOVA for PPI revealed significant main effects of EGF dose ($F_{(3,30)} = 5.58$; $p = 0.0036$) and prepulse amplitude ($F_{(2,60)} = 58.8$; $p < 0.0001$) that were independent. *Post hoc* analysis indicated that the maximum dose of EGF (30 μg/pump) significantly reduced PPI with 75 and 85 dB prepulse tones. Therefore, EGF administered to the striatum elevated dopamine turnover and reduced PPI in a dose-dependent manner. In subsequent experiments, rats received the highest dose of EGF (30 μg/pump, 1.8 μg/d).

Intrastriatal administration of EGF induces Cox-2 immunoreactivity

After subchronic EGF infusion, distributions of EGF in the brain were examined by immunohistochemistry with an anti-EGF antibody. EGF appeared to diffuse through the striatal region efficiently. There was marked EGF immunoreactivity in the ipsilateral striatum as well as in the somatosensory neocortex along the route of the cannula (Fig. 2*A*). No immunoreactivity for EGF was detected in animals receiving an infusion of saline (data not shown). Because EGF is a potent Cox-2 inducer (Slice et al., 2005; Liao et al., 2006), we also examined Cox-2 immunoreactivity in EGF-infused rats. EGF increased the immunoreactivity of Cox-2 in the striatum and around the ventricular wall, relative to vehicle-infused animals (Fig. 2*B–E*).

Effects of EGF depletion and bilateral EGF administration on PPI

To examine the reversibility of the effect of EGF on PPI, first we confirmed its effects by administering rats EGF for 7 d and then

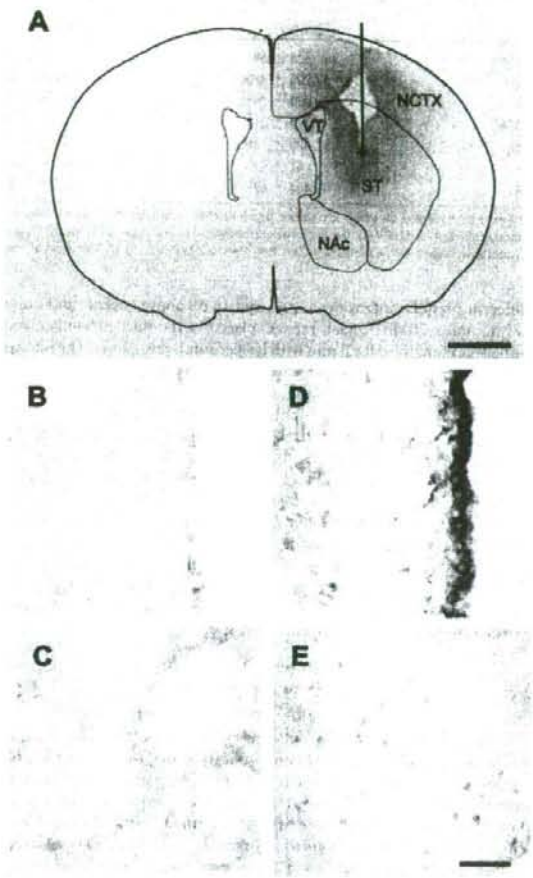

Figure 2. Distributions of EGF and Cox-2 immunoreactivity after subchronic striatal administration of EGF. ***A***, The efficacy of EGF infusion was examined by immunohistochemistry. EGF was administered to the striatum of adult male rats from an osmotic minipump (30 μg/pump) for 8 d. Coronal sections along the cannula route were immunostained with an anti-human EGF antibody. ***B–E***, The distribution of Cox-2 immunoreactivity was examined in vehicle-infused (***B***, ***C***) and EGF-infused (***D***, ***E***) animals. The most marked increase in Cox-2 immunoreactivity was observed around the lateral ventricle (***D***) and in the striatum (***E***) of the ipsilateral hemisphere. NCTX, Neocortex; ST, striatum; VT, lateral ventricle; NAc, nucleus accumbens. Scale bars: ***A***, 250 μm; ***B–E***, 30 μm. An arrow indicates the cannula position.

monitoring acoustic startle responses to 120 dB noise and PPI (Fig. 3). EGF infusion did not alter the startle response to 120 dB noise when compared with vehicle-infused animals (two tailed t test, $p = 0.072$) (Fig. 3*A*). We confirmed the significant main effects of EGF ($F_{(1,28)} = 6.59$; $p = 0.016$) on PPI (Fig. 3*C*). *Post hoc* analysis detected a significant decrease in PPI levels with the 85 dB prepulse tone. Ten days after cessation of EGF administration, there was no significant difference in startle amplitudes and PPI levels between EGF-treated and vehicle-treated animals, demonstrating that the effects of EGF are reversible (Fig. 3*B*,*D*).

To examine whether the effect on PPI were a consequence of unilateral infusion of EGF, we assessed bilateral effects of EGF infusion on PPI. We subchronically administered EGF to the striatum in both hemispheres from two osmotic minipumps and measured acoustic startle responses to 120 dB noise (Fig. 4*A*,*B*).

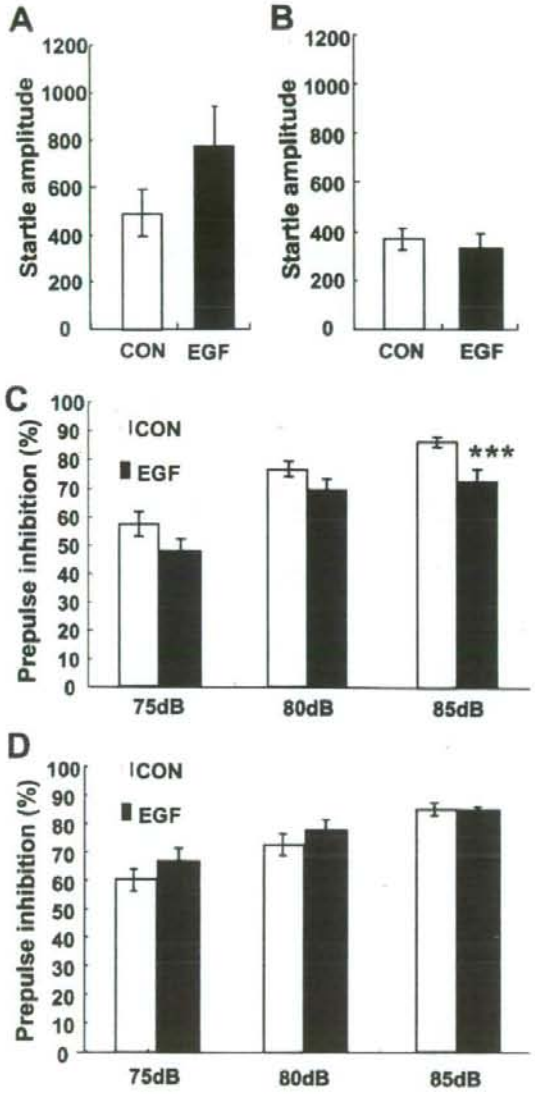

Figure 3. Effects of EGF depletion on acoustic startle response and prepulse inhibition. ***A***, ***B***, Acoustic startle response of vehicle-infused control (CON; open box) and EGF-infused (filled box) rats was measured on day 8 of EGF administration (30 μg/pump) (***A***) and 10 d after completion of EGF administration (***B***). ***C***, ***D***, Simultaneously, PPI of vehicle-infused control (open box) and EGF-infused (filled box) animals with 75, 80, and 85 dB prepulse stimuli was measured during EGF administration (***C***) and after completion of EGF administration (***D***). Error bars indicate means ± SEM ($n = 15$ each). ***$p < 0.001$ by Fisher's LSD.

EGF infusion to the striatum in both hemispheres did not significantly alter the startle response to 120 dB noise when compared with vehicle-infused animals. A two-way repeated ANOVA revealed significant main effects of bilateral EGF infusion ($F_{(1,10)} = 7.58$; $p = 0.020$) and prepulse amplitude ($F_{(2,20)} = 19.5$; $p < 0.001$) on PPI without interaction. *Post hoc* analysis identified significant decreases in PPI with the 75 and 80 dB prepulse tones.

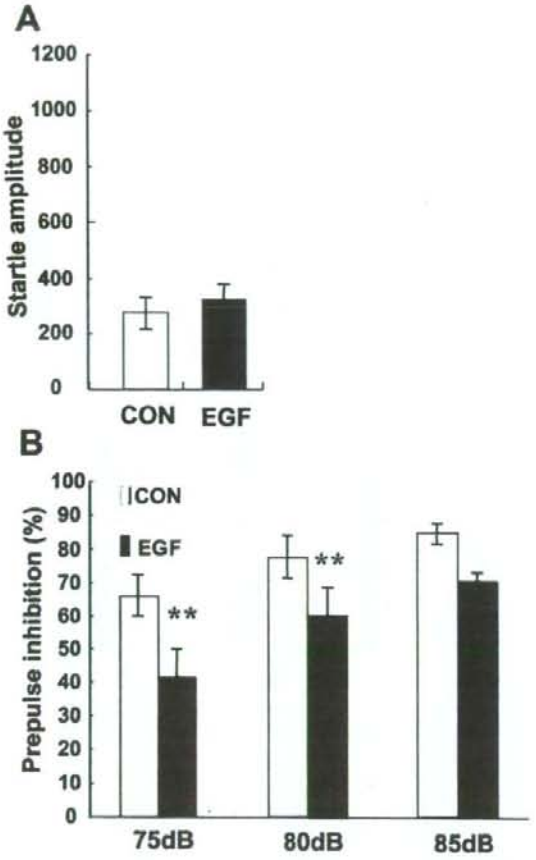

Figure 4. Effects of bilateral EGF infusion on acoustic startle response and PPI. EGF was administered to both hemispheres of the striatum of adult rats from two osmotic minipumps (30 μg/pump × 2). ***A***, Acoustic startle response of vehicle-infused control (CON; open box) and EGF-infused (filled box) rats was measured 8 d after bilateral EGF administration was initiated. ***B***, Simultaneously, PPI of vehicle-infused control (open box) and bilaterally EGF-infused (filled box) animals with 75, 80, and 85 dB prepulse stimuli was measured. Error bars indicate means ± SEM ($n = 6$ each). **$p < 0.01$ by Fisher's LSD.

Effects of EGF administration to the nucleus accumbens on prepulse inhibition

We also monitored the subchronic effects of EGF administration to the nucleus accumbens, a locus most implicated in PPI regulation (Swerdlow et al., 1990, 2001; Swerdlow and Geyer, 1998). Immunohistochemistry revealed that EGF immunoreactivity was predominantly localized around the nucleus accumbens as well as along the cannula route (Fig. 5*A*). There was no significant difference in acoustic startle responses between saline- and EGF-infused rats (Fig. 5*B*). A two-way repeated ANOVA of PPI scores revealed no main effect of EGF treatment but a significant effect of prepulse amplitude ($F_{(2,28)} = 47.0$; $p < 0.0001$) without their interaction (Fig. 5*C*). Thus, the administration of EGF to the nucleus accumbens failed to influence PPI levels.

Effects of intrastriatal EGF infusion on neurochemical markers

To assess the neurochemical consequences of striatal EGF infusion, we examined molecular markers for EGF signaling as well as

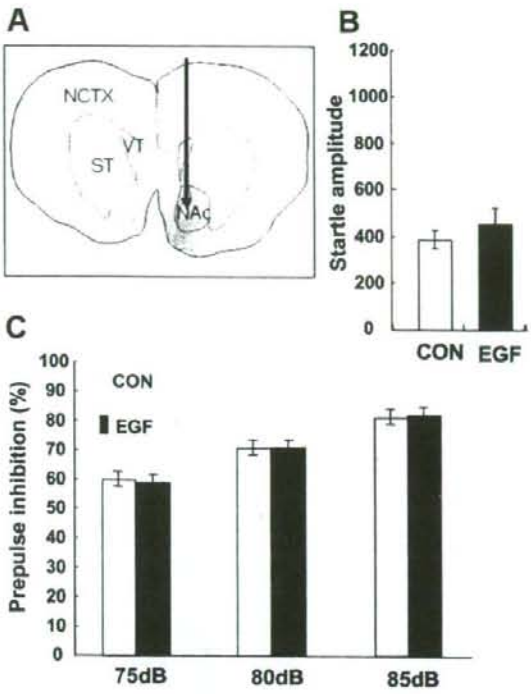

Figure 5. Effects of EGF infusion into the nucleus accumbens on PPI. EGF was administered to the nucleus accumbens of adult male rats from an osmotic minipump (30 μg/pump) for 8 d. ***A***, The efficacy of EGF infusion to the nucleus accumbens was examined by immunohistochemistry. Coronal sections along the cannula route were immunostained with an anti-human EGF antibody. ***B***, Acoustic startle response of vehicle-infused control (CON; open box) and EGF-infused (filled box) rats was measured before fixation for the above immunohistochemistry. PPI of vehicle-infused control and EGF-infused animals was determined with 75, 80, and 85 dB prepulse stimuli (***C***). Error bars indicate means ± SEM (n = 11 each). NCTX, Neocortex; ST, striatum; VT, lateral ventricle; NAc, nucleus accumbens. An arrow indicates the cannula position.

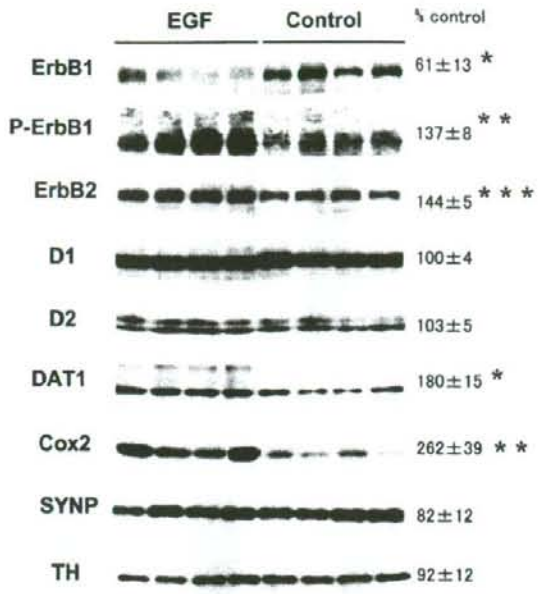

Figure 6. ErbB1 phosphorylation and neurochemical markers induced by EGF administration. EGF (30 μg/pump) or saline was administered into the striatum of adult rats (n = 4 animals each). On day 12 of the EGF infusion, protein extract was prepared from the ipsilateral striatum and subjected to immunoblotting for antibodies directed against ErbB1, phosphorylated ErbB1, ErbB2, DAT, D_1 receptor, D_2 receptor, synaptophysin (SYNP), tyrosine hydroxylase (TH), and Cox-2. Immunoreactivity was measured by densitometric analysis, and its ratio to that in vehicle-infused control rats (mean ± SEM) is presented. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two-tailed t test (n = 4 animals each).

those for dopamine signaling in the striatum (Fig. 6). EGF administration significantly elevated phosphorylation levels of ErbB1 (to 137%; two-tailed t test, $p < 0.001$) and conversely downregulated total protein levels of ErbB1 (to 61%; $p = 0.039$). As indicated in the above immunohistochemistry, EGF administration upregulated total protein levels of Cox-2 (to 262%; $p = 0.006$). In parallel, DAT levels were increased (179%; $p = 0.041$), although there were no significant changes in the expression of dopamine receptors, the synaptic marker synaptophysin, or tyrosine hydroxylase (Fig. 6).

A Cox-2 inhibitor ameliorates the EGF-induced deficit of prepulse inhibition

To evaluate the contribution of Cox-2 induction to the PPI deficit, rats were simultaneously given a Cox-2 inhibitor, celecoxib (10 mg/kg, p.o.). Prepulse inhibition of vehicle-infused and EGF-infused animals was examined 1 h and 7 d after celecoxib or saline treatment was initiated (Fig. 7). In the acute paradigm of celecoxib administration, the effect of the Cox-2 inhibitor on PPI was analyzed by three-way ANOVA using between-subject factors of EGF [in its absence or presence (+/−)] and celecoxib (+/−) and a within-subject factor of prepulse intensity (Fig. 7*A*). Celecoxib failed to exhibit a main effect on PPI levels, although main effects of EGF ($F_{(1,26)} = 18.9$; $p < 0.001$) and prepulse intensity ($F_{(2,52)} = 63.7$; $p < 0.001$) were significant. There were no factorial interactions. Similarly, the subchronic effects of celecoxib administration were evaluated by three-way ANOVA. Although there were significant main effects of EGF ($F_{(1,17)} = 8.59$; $p = 0.009$) and celecoxib ($F_{(1,17)} = 9.39$; $p = 0.007$), ANOVA detected a significant interaction between EGF and celecoxib ($F_{(1,17)} = 11.2$; $p = 0.004$), suggesting that the therapeutic effects of celecoxib significantly differ between EGF-infused and vehicle-infused animals. Accordingly, the effects of celecoxib were separately evaluated in either the vehicle-infused or EGF-infused group. ANOVA revealed no significant main effect of celecoxib in the vehicle-infused group (Fig. 7*B*). In contrast, celecoxib administration to EGF-infused animals improved PPI scores significantly ($F_{(1,13)} = 2.61$; $p < 0.001$) (Fig. 7*C*).

Effects of celecoxib treatment on fear learning and its latent inhibition of EGF-infused rats

We also evaluated the effects of striatal EGF infusion and celecoxib on fear learning and latent inhibition. EGF- and vehicle-infused rats were subjected to an active-avoidance test with a two-way shuttle chamber in the presence or absence of preexposure to a buzzer tone (CS) (Salmi et al., 1994). Initial four-way ANOVA using between-subject factors of EGF infusion (+/−), celecoxib administration (+/−), and preexposure to CS (+/−) and a within subject factor of test block (six) revealed significant interactions between EGF and preexposure ($F_{(1,72)} = 7.65$; $p = 0.007$) and between pre-exposure and block ($F_{(5,360)} = 5.27$; $p <$

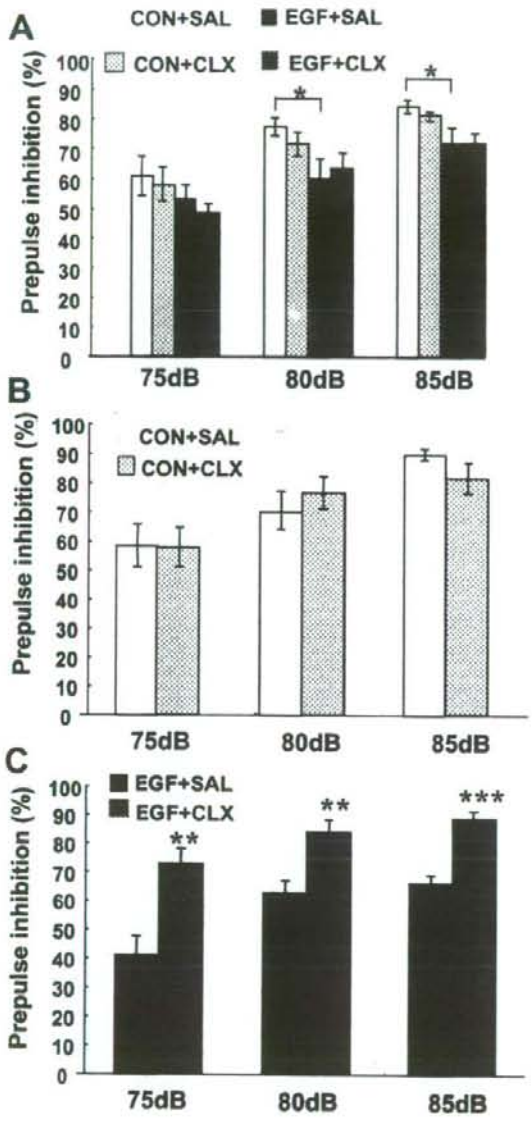

Figure 7. Effects of a Cox-2 inhibitor on the PPI deficits of rats receiving EGF in the striatum. Three days after striatal administration of EGF (30 μg/pump) or vehicle (CON) was initiated, rats were orally given celecoxib (CLX) or saline (SAL) daily. ***A***, One hour after the first dose of celecoxib, PPI of the acoustic startle response with 75, 80, and 85 dB prepulse stimuli was measured. ***B***, ***C***, At 7 d of treatment with celecoxib or saline, PPI was measured in vehicle-infused (***B***) and EGF-infused (***C***) rats. White and black bars represent vehicle-infused and EGF-infused rats that received saline orally. Black dotted and white dotted bars represent vehicle-infused and EGF-infused rats that received celecoxib orally. Error bars indicate means ± SEM for each prepulse intensity ($n = 7$–8 each). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with saline-infused controls (***A***) or EGF-infused rats receiving saline (***C***) by Fisher's LSD. Note that the subchronic effects of celecoxib were measured at least 20 h after the last treatment with celecoxib.

0.001). No other interactions were observed among between-subject factors. The statistical interactions indicated that EGF differentially affected the latent learning scores and that the preexposure effects varied during test sessions. Accordingly, behavioral scores were separated into EGF-infused and vehicle-infused groups and subjected to statistical analysis independently.

A three-way-ANOVA of learning data from the vehicle-infused group revealed a significant main effect of preexposure ($F_{(1,36)} = 22.9$; $p < 0.001$) without any interaction of between-subject factors. The significant interaction between preexposure and block indicated that the effect of preexposure differed among blocks ($F_{(5,180)} = 3.79$; $p = 0.027$) (Fig. 8*A*). *Post hoc* comparisons detected a significant effect of preexposure on learning regardless of celecoxib treatment in blocks 4–6 (Fig. 8*A*). In contrast, the same analysis for EGF-infused group revealed a significant main effect of preexposure ($F_{(1,36)} = 10.7$; $p = 0.023$), marginal interactions between preexposure and celecoxib ($F_{(1,36)} = 3.31$; $p = 0.077$), and marginal interactions between preexposure and session ($F_{(5,180)} = 1.98$; $p = 0.083$). Pillai compensation for repeated measures confirmed statistical significance of the interaction between celecoxib and block ($F_{(5,180)} = 2.693$; $p = 0.039$). *Post hoc* comparisons revealed that celecoxib treatment significantly improved learning scores of the preexposure group but not the non-preexposure group in block 6 compared with the vehicle-treated non-preexposure group (Fig. 8*B*). Neither EGF infusion nor celecoxib treatment appeared to alter locomotion as monitored by the number of intershuttle movements during the intertrial periods (see details in legend of Fig. 8). We focused on behavioral performance during the last test session and calculated the decrease in learning score that was caused by preexposure to the test chamber without a shock (Fig. 8*C*). A two-way ANOVA with subject factors of EGF administration (+/−) and celecoxib treatment (+/−) revealed significant interactions between EGF and celecoxib for this latent inhibition score ($F_{(1,18)} = 13.1$; $p = 0.002$). *Post hoc* comparisons confirmed that striatal EGF infusion disrupted the latent inhibition of fear learning ($p = 0.002$), and subchronic treatment of celecoxib ameliorated the abnormal decrease in latent inhibition ($p = 0.002$).

To assess the effect of EGF infusion on basal fear learning performance, behavioral data were separated into non-preexposed and preexposed groups to avoid the statistical interaction. In non-preexposed group, three way-ANOVA using between-subject factors of EGF infusion (+/−) and celecoxib administration (+/−) and a within-subject factor of test block (six) revealed that there was not a significant main effect of EGF or celecoxib, and there was no interaction of between-subject factors.

To confirm the ineffectiveness of EGF and celecoxib on basal learning performance and shock sensitivity, we performed another fear learning test (Fig. 9). EGF- and vehicle-infused rats, which were treated with saline or celecoxib, were subjected to contextual conditioning with electric shocks (CS) and environmental context (US). Two-way ANOVA failed to detect a significant difference in shock sensitivity among groups without interaction (Fig. 9*A*). Learning performance was also indistinguishable among groups (Fig. 9*B*). Thus, we conclude that neither striatal EGF infusion nor celecoxib treatment influence basal learning performance or shock sensitivity.

EGF activates dopamine synthesis and turnover

Deficits in PPI and latent inhibition are proposed to involve abnormal dopaminergic neurotransmission (Swerdlow et al., 2001; Jeanblanc et al., 2003; Smith et al., 2007). To evaluate the contribution of Cox-2 induction to EGF-enhanced dopamine turnover, we also examined the effects of the Cox-2 inhibitor celecoxib on striatal concentrations of dopamine and its metabolites (Fig. 10). A two-way ANOVA with subject factors of EGF admin-

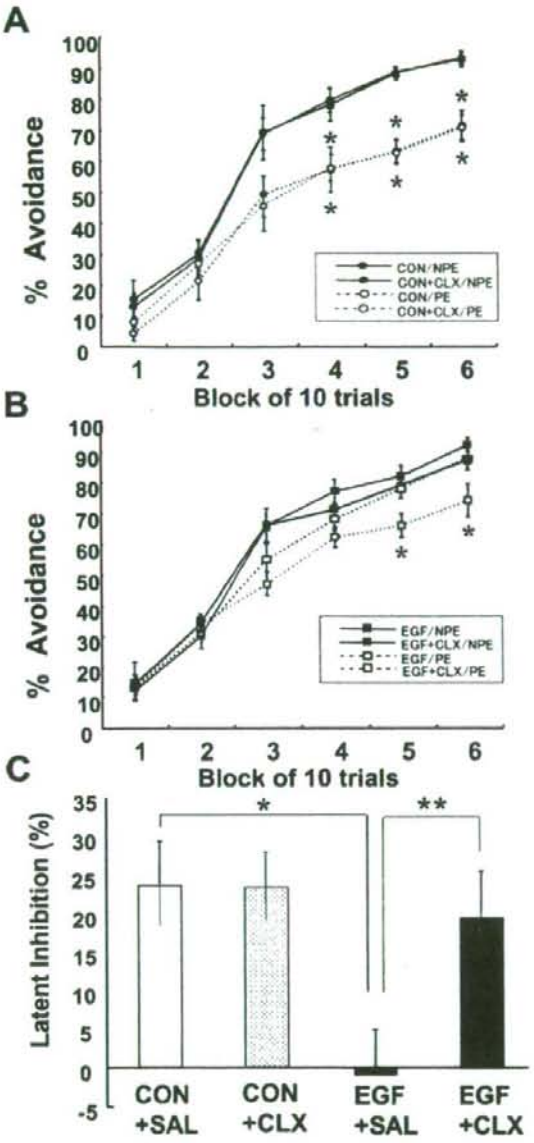

Figure 8. The effects of celecoxib on active-avoidance learning and latent inhibition in EGF-infused rats. Learning ability and latent inhibition of EGF-infused rats (EGF; 30 μg/pump) was determined with an active-avoidance test (10 trials per block, 6 blocks total) and compared with that of vehicle-infused controls (CON). Simultaneously, these animals were daily treated with celecoxib (CLX) or saline (SAL). Detailed schedules of the EGF infusion and celecoxib are described in Materials and Methods. The ability of vehicle- and EGF-infused rats to avoid the electrical shock paired with a tone was defined as learning performance and improved significantly during training sessions. Before the conditioning, rats in the PE group had been preexposed to the same tone cues without shock. Rats in the NPE group were directly subjected to the active-avoidance test. Random shuttle movement of the NPE group during intertrial periods revealed no effects of EGF by ANOVA or celecoxib or factorial interaction. ***A***, ***B***, Because the initial four-way ANOVA revealed factorial interaction between EGF infusion and preexposure, data were separated into vehicle-infused (***A***) and EGF-infused (***B***) groups and then analyzed separately. ***C***, Learning performance at the last block (6th) of the PE groups was compared with that of the NPE group and latent inhibition scores were calculated as follows:

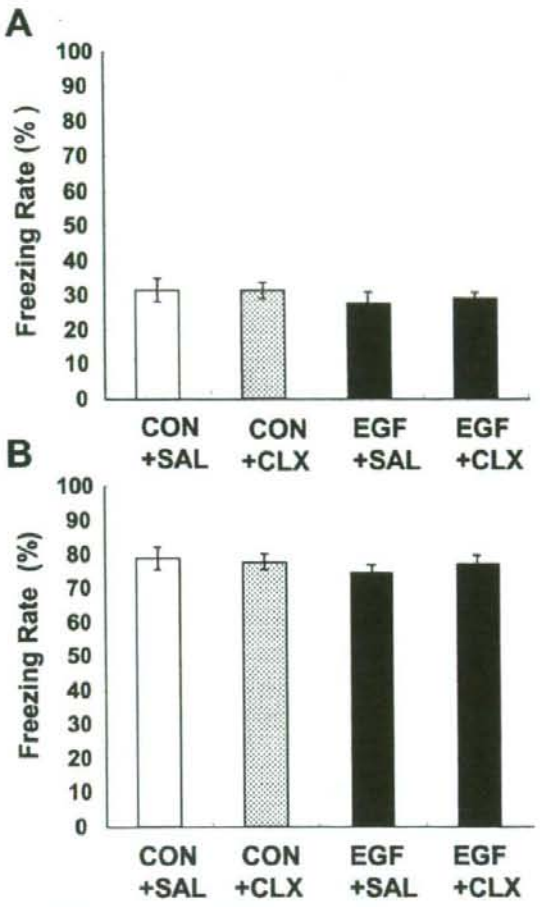

Figure 9. Effects of EGF and celecoxib on shock sensitivity and context learning. Three days after striatal administration of EGF (30 μg/pump) or vehicle (CON) was initiated, rats were orally given celecoxib (CLX) or saline (SAL) daily for 7 d and subjected to conditioning. ***A***, Immobilizing rate (freezing) of rats was calculated for 2 min after the second shock of conditioning. ***B***, Contextual fear learning was evaluated with freezing rates from 1 to 3 min after placing rats in the same chamber 1 d after conditioning. White and black bars represent vehicle-infused and EGF-infused rats that received saline, respectively. Black dotted and white dotted bars represent vehicle-infused and EGF-infused rats that received celecoxib, respectively. Error bars indicate means ± SEM ($n = 10$ each). Note that conditioning was performed 24 h after the last treatment with celecoxib.

istration (+/−) and celecoxib treatment (+/−) revealed significant and marginal interactions between EGF and celecoxib for dopamine and its metabolites, respectively ($F_{(1,17)} = 4.43$, $p = 0.050$ for dopamine; $F_{(1,17)} = 3.99$, $p = 0.062$ for DOPAC; $F_{(1,17)} = 11.2$, $p = 0.039$ for HVA). *Post hoc* comparisons revealed that elevated dopamine and HVA contents after EGF administra-

←

$$\text{Latent inhibition score} = 100 - \frac{(\text{performance of each PE rat}) \times 100}{\text{mean performance of NPE group}}.$$

Error bars indicate means ± SEM ($n = 10$ each). *$p < 0.05$, **$p < 0.01$ by Fisher's LSD. Note that, to minimize acute effects of celecoxib treatment, the active-avoidance test was performed at least 20 h after the last treatment with celecoxib.