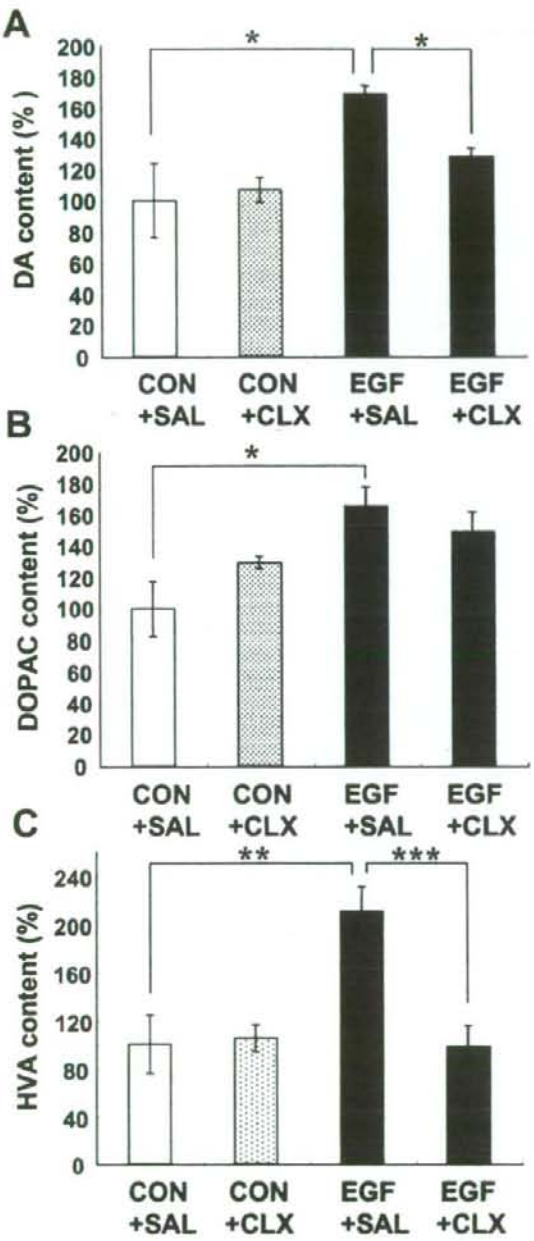

Figure 10. Effects of subchronic celecoxib treatment on dopamine and its metabolites. The levels of dopamine and its metabolites were measured in the striatum of vehicle-infused (CON) and EGF-infused (EGF; 30 μg/pump) control rats that also subchronically received celecoxib (CLX) or saline (SAL). Note that, to minimize acute effects of celecoxib treatment, tissue dissection was performed at least 20 h after the last treatment with celecoxib. White and black bars represent vehicle-infused and EGF-infused rats receiving saline orally. Black dotted and white dotted bars represent vehicle-infused and EGF-infused rats receiving celecoxib orally. Error bars indicate means ± SEM ($n = 5–6$ each). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Fisher's LSD.

Table 2. Effects of EGF infusion and celecoxib on tyrosine hydroxylase activity in the striatum

	Tyrosine hydroxylase activity		
	CON-SAL	EGF-SAL	EGF-CLX
Dorsal striatum	9.8 ± 0.5	12.1 ± 0.5*	12.3 ± 0.8*
Ventral striatum	9.4 ± 1.0	12.5 ± 0.6*	11.7 ± 0.6*

EGF (30 μg/pump) or saline (CON) was subchronically administered to the striatum of rats. Some rats were orally given celecoxib (CLX) or saline (SAL) 3 d after EGF infusion was initiated. The celecoxib treatment was done daily and repeated for 7 d. Tissue homogenates were prepared from the ipsilateral hemisphere of the striatum, and the activity of tyrosine hydroxylase was measured by monitoring the production of L-DOPA. Data represent means ± SEM (picomoles per milligram of protein; $n = 6$ animals each). *$p < 0.05$ compared with saline-infused control rats (CON-SAL) by Fisher's LSD.

tion were significantly lowered by subchronic treatment with celecoxib.

How does striatal EGF infusion trigger dopamine-associated behavioral deficits and elevate dopamine levels without changing the expression of the rate limiting enzyme for dopamine synthesis, tyrosine hydroxylase? There are reports that inflammatory cytokines including EGF can elevate the activity of tyrosine hydroxylase by phosphorylating the enzyme or upregulating its cofactor tetrahydrobiopterin (Halegoua and Patrick, 1980; Anastasiadis et al., 1997). We measured the tyrosine hydroxylase activity in the dorsal and ventral striatum of the rats receiving EGF in the striatum (Table 2). The activity of tyrosine hydroxylase in the dorsal striatum of EGF-infused animals was significantly larger than that of saline-infused controls ($p = 0.017$). An increase in the enzymatic activity was also detected in the ventral striatum ($p = 0.012$). However, subchronic cotreatment with celecoxib did not attenuate the increase in the activity of tyrosine hydroxylase. We conclude that the increase in dopamine content observed after EGF infusion is, in part, attributable to increased enzymatic activity of tyrosine hydroxylase.

Discussion

The alterations in inflammatory cytokine levels and the effectiveness of anti-inflammatory drugs observed in schizophrenia patients are consistent with the neuroinflammatory hypothesis of schizophrenia (Muller et al., 2000; Nawa and Takei, 2006). Here we combined the evidence that EGF is an inducer for Cox-2 expression (Ackerman et al., 2004; Slice et al., 2005) with our previous finding that EGF signaling might be upregulated in the striatum of schizophrenia patients (Futamura et al., 2002) and hypothesized that enhanced EGF signaling might increase prostaglandin synthesis and lead to behavioral deficits. Therefore, we tested the neurobehavioral consequences of EGF stimulation in rat striatum as well as the antagonistic effects of Cox-2 inhibition. Subchronic infusion of EGF and concomitant celecoxib treatment produced the following results: (1) striatal infusion of EGF yields behavioral deficits in PPI and latent inhibition of fear learning; (2) these deficits were reversible and extinguished by cessation of EGF infusion; (3) EGF administration elevated the expression of Cox-2, the enzyme activity of tyrosine hydroxylase, and dopamine turnover in the striatum; and (4) subchronic treatment with a Cox-2 inhibitor ameliorated these behavioral deficits and concomitantly normalized dopamine turnover. These observations strengthen the argument that EGF-mediated neuroinflammation may, at least in part, result in abnormal dopamine transmission and associated behavioral deficits.

Enhanced EGF signaling in the striatum and behavioral impairments

The present findings might illuminate the neuropathological implication of abnormal levels of EGF or its receptor in the brain of

schizophrenia patients (Futamura et al., 2002). The postmortem study demonstrates that, among various ErbB1 ligands, EGF content is specifically decreased in the striatum of schizophrenia patients. Whether this decrease in EGF content reflects enhanced release of EGF from vesicular stores or decreased EGF synthesis is not clear. To address this question, here, we exogenously supplied EGF to the striatum: subchronic infusion of EGF into the striatum induced behavioral impairments. Gross learning ability of EGF-infused rats was normal in active-avoidance test (Fig. 8) as well as in contextual fear conditioning (Fig. 9), however. Abnormalities in PPI as well as in latent inhibition are often observed in schizophrenia patients (Gray et al., 1995; Weiner and Feldon, 1997; Braff et al., 2001; Swerdlow et al., 2001). Accordingly, these results suggest that elevated EGF/ErbB1 signaling in the striatum might contribute to etiology or pathology of schizophrenia.

EGF and other ErbB1 ligands elevate the expression of tyrosine hydroxylase (Casper et al., 1994; Farkas et al., 2002; Iwakura et al., 2005). EGF also modulates the activity of tyrosine hydroxylase by promoting phosphorylation of the enzyme or increasing the synthesis of its essential cofactor tetrahydrobiopterin (Halegoua and Patrick, 1980; Anastasiadis et al., 1997). There was a significant increase in its enzymatic activity in EGF-infused animals. Because this enzyme limits the rate of dopamine synthesis, this increase in enzymatic activity presumably led to an increase in the synthesis and release of dopamine. In this context, the increase in DAT expression might result from enhanced dopamine release through the negative feedback regulation (Xia et al., 1992; Fang and Ronnekleiv, 1999).

Subcutaneous administration of EGF to neonatal rats and mice increases dopamine turnover and later results in life-long neurobehavioral deficits (Futamura et al., 2003; Tohmi et al., 2005). In that experimental paradigm, however, there is a large time lag between the dopaminergic abnormality in neonates and the emergence of the behavioral impairments in adults. Here we learned that striatal EGF infusion to adult animals similarly perturbed dopaminergic responses and mimicked the behavioral deficits induced by neonatal treatment with EGF. Thus, the behavioral deficits induced by neonatal EGF treatment might share a common pathologic mechanism with those of the present striatal EGF infusion, although the persistency of the deficits differs significantly.

Behavioral deficits associated with elevated dopamine synthesis and metabolism

Consistent with these reports, the present experiments demonstrate that EGF-triggered behavioral abnormalities are concomitant with changes in dopaminergic metabolism in the striatum. EGF administration increases the concentrations of dopamine and its metabolites in the striatum and impaired prepulse inhibition performance in a dose-dependent manner, and, conversely, celecoxib treatment normalized the levels of this neurotransmitter and metabolites, as well as behavioral performance. This elevated dopamine metabolism, but not the increase in the tyrosine hydroxylase activity, was reversed by subchronic treatment with the Cox-2 inhibitor celecoxib. Previous reports demonstrated that prostaglandins influence the activity of excitatory neurons and monoaminergic neurons (Takechi et al., 1996; Oida et al., 1997; Nakamura et al., 2001; Matsuoka et al., 2005; Sang et al., 2005). Given the defined roles of EGF and prostaglandin, we speculate that EGF may increase dopamine synthesis and prostaglandins may enhance its release. These two effects may act synergistically to induce the observed behavioral deficits, although these explanations need to be verified in future experiments including *in vivo* microdialysis.

Targets of EGF in the brain

Lesion studies indicate a major contribution of inhibitory striatopallidal circuitry to the acoustic startle reflexes regulating PPI (Swerdlow et al., 1990, 2001). Unilateral dysfunction of this inhibitory circuitry appears to be sufficient to impair PPI responses (Li et al., 1998; Uehara et al., 2007). In agreement with these reports, both unilateral and bilateral infusion of EGF similarly decreased PPI in the present study.

Enhanced dopamine signaling in both ventral and dorsal striatum is implicated in PPI deficits (Kodsi and Swerdlow, 1995; Swerdlow et al., 2001). Among the striatal regions, the nucleus accumbens is suggested to play an important role in regulating PPI (Swerdlow et al., 1990, 2001). Even when EGF was injected to the center of the striatum, EGF immunoreactivity and an increase in the tyrosine hydroxylase activity were also present in the ventral striatum, including the nucleus accumbens. Accordingly, we had expected that EGF infusion directly into the nucleus accumbens would similarly affect PPI. However, it was not the case. We speculate the reasons. Implanting the cannula directly into the nucleus accumbens might produce surgical injury, potentially counteracting the EGF action or perturbing the local neurotransmission. Alternatively, our preliminary result that dopaminergic terminals in the nucleus accumbens express lower levels of ErbB1 may account for the discrepancy (Zheng et al., 2007). Because latent inhibition of learning involves various brain regions and neural circuits including the striatum, the nucleus accumbens, the limbic system, and the cholinergic system (Weiner and Feldon, 1997; Jeanblanc et al., 2003; Peterschmitt et al., 2005), understanding the present discrepancy may require future experiments of greater complexity.

Neurobehavioral and antipsychotic effects of the Cox-2 inhibitor celecoxib

Significant basal levels of Cox-2 are detectable in several brain regions (Tsubokura et al., 1991; Yamagata et al., 1993; Kaufmann et al., 1996). Inflammatory cytokines induce Cox-2 expression after brain injury, ischemia, and hypoxia (Smith et al., 2000; Ackerman et al., 2004). Increasing Cox-2 expression elevates the levels of all five prostaglandins and activates their downstream receptor signaling (Smith et al., 2000). Conversely, Cox-2 inhibitors attenuate post-ischemic cell death or neurodegeneration associated with Alzheimer's disease (Firuzi and Pratico, 2006). Thus, Cox-2 induction and resultant prostaglandin synthesis are often implicated in neurodegeneration, although the neurotrophic and neurodegenerative actions of prostaglandins remain controversial (Hewett et al., 2000; Strauss and Marini, 2002; Liang et al., 2005). The transgenic mouse model for Alzheimer's disease that overexpresses Cox-2 exhibits age-associated working memory deficits and spatial memory impairment, both of which are sensitive to Cox inhibitors (Sharifzadeh et al., 2005; Melnikova et al., 2006). However, the EGF-induced cognitive deficits in the present study do not appear to involve neurodegeneration because markers for neurons and synapses and brain histochemistry were not altered, the behavioral deficits were reversible, and gross learning scores were normal.

There are several reports demonstrating that the antipsychotic effects and neurocognitive improvement are associated with administration of Cox inhibitors (Ho et al., 2006). Add-on therapy of the Cox-2 inhibitor celecoxib to risperidone improves PANSS of patients with schizophrenia compared with those treated with

risperidone alone (Muller et al., 2004; Riedel et al., 2005). A follow-up study indicates the most pronounced therapeutic effects of Cox-2 inhibitors are cognitive improvements (Riedel et al., 2005). Nonspecific Cox inhibitors such as indomethacin and piroxicam also reverse behavioral and cognitive deficits induced by cocaine, amphetamines, and brain inflammation (Reid et al., 2002; Ross et al., 2002; Matsumoto et al., 2004). The present study may reveal aspects of the mechanism underlying the effectiveness of this medication protocol. Oral administration of celecoxib for 1 week normalized PPI deficits, whereas a single oral dose failed to do so. Thus, subchronic suppression of Cox-2 activity is required to exert significant effects in this animal model (Rivest, 1999; Carothers et al., 2006). In addition, there was no significant effect of celecoxib on basal PPI levels in control animals, suggesting that basal Cox-2 expression, which is normally present in the corticolimbic system (Tsubokura et al., 1991; Yamagata et al., 1993; Kaufmann et al., 1996), do not influence PPI. Although it remains to be determined which types of prostaglandins contribute to the behavioral and cognitive impairments observed after EGF administration, these experiments support the hypotheses that, in addition to its role in neurological diseases, inflammation-triggered prostaglandin synthesis and signaling are potential therapeutic targets for schizophrenia and related psychiatric disorders.

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Identification of *YWHAE*, a gene encoding 14-3-3epsilon, as a possible susceptibility gene for schizophrenia

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Schizophrenia is a complex mental disorder with a fairly high degree of heritability. Although the causes of schizophrenia remain unclear, it is now widely accepted that it is a neurodevelopmental and neurodegenerative disorder involving disconnectivity and disorder of the synapses. Disrupted-in-schizophrenia 1 (*DISC1*) is a promising candidate susceptibility gene involved in neurodevelopment, including maturation of the cerebral cortex. To identify other susceptibility genes for schizophrenia, we screened for *DISC1*-interacting molecules [NudE-like (*NUDEL*), Lissencephaly-1 (*LIS1*), 14-3-3epsilon (*YWHAE*), growth factor receptor bound protein 2 (*GRB2*) and Kinesin family 5A of Kinesen1 (*KIF5A*)], assessing a total of 25 tagging single-nucleotide polymorphisms (SNPs) in a Japanese population. We identified a *YWHAE* SNP (*rs28365859*) that showed a highly significant difference between case and control samples, with higher minor allele frequencies in controls ($P_{allele} = 1.01 \times 10^{-5}$ and $P_{genotype} = 4.08 \times 10^{-5}$ in 1429 cases and 1728 controls). Both messenger RNA transcription and protein expression of 14-3-3epsilon were also increased in the lymphocytes of healthy control subjects harboring heterozygous and homozygous minor alleles compared with homozygous major allele subjects. To further investigate a potential role for *YWHAE* in schizophrenia, we studied *Ywhae*$^{+/-}$ mice in which the level of 14-3-3epsilon protein is reduced to 50% of that in wild-type littermates. These mice displayed weak defects in working memory in the eight-arm radial maze and moderately enhanced anxiety-like behavior in the elevated plus-maze. Our results suggest that *YWHAE* is a possible susceptibility gene that functions protectively in schizophrenia.

INTRODUCTION

Recent neuroimaging studies show that structural brain abnormalities are an established feature of schizophrenia and are characterized by decreased total gray matter volume (1,2). These morphological correlates of schizophrenia range from a reduction in brain size to localized alterations in the morphology and molecular composition of specific neuronal, synaptic and glial populations in specific brain areas such as the hippocampus, dorsolateral prefrontal cortex and dorsal thalamus.

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†The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First authors.

These findings have fostered the current view of schizophrenia as a disorder of connectivity (3,4) and of the synapse (5). Although the mechanism underlying the neurodevelopmental/neurodegenerative process is still unclear, a way forward is provided by the recent identification of several putative susceptibility genes, such as *Neuregulin 1* (6), *Dysbindin* (7), *G72* (8), Catechol-*O*-methyltransferase (*COMT*) (9–11) and others (12,13). For none of these genes, however, has a causative allele or the mechanism by which it predisposes to schizophrenia been identified.

Disrupted-in-schizophrenia 1 (*DISC1*) was first described as a strong candidate gene in a large Scottish family in which a balanced chromosomal translocation segregates with schizophrenia and other psychiatric disorders (12,14,15). The translocation mutation may result in loss of DISC1 function via haploinsufficiency or dominant-negative effects of a predicted mutant DISC1 truncated protein product. *DISC1* has been implicated in neurodevelopment, including maturation of the cerebral cortex (16).

DISC1 interacts with several proteins, including NudE-like (NUDEL) (17–19), lissencephaly-1 (LIS1, also called PAFAH1B1) (20), fasciculation and elongation protein zeta 1 (FEZ1) (21) and phosphodiesterase 4B (PDE4B) (16). Recently, we identified several novel DISC1-interacting molecules, including 14-3-3epsilon, Kinesin family 5A of Kinesen1 (KIF5A) and Growth factor receptor bound protein 2 (Grb2) by affinity column chromatography (22,23). Furthermore, we confirmed that DISC1 regulates the localization of the NUDEL/LIS1/14-3-3epsilon complex or Grb2 into axons as a cargo receptor (22,23) and it also regulates Neurotrophin-induced axon elongation by Grb2 (23).

In this study, we screened for the genetic association of DISC1-interacting molecules—*NUDEL* (17p13.1, OMIM: *607538), *LIS1* (17p13.3, OMIM: #607432), 14-3-3epsilon (17p13.3, OMIM: *605066), *Grb2* (17p24-q23, OMIM: *108355) and *KIF5A* (12q13, OMIM: *602821)—with schizophrenia, and identified the gene encoding 14-3-3epsilon (*YWHAE*) as a possible susceptibility gene. Our results show that a SNP of *YWHAE*, which influence the expression of 14-3-3epsilon RNA and protein, is associated with schizophrenia and seems to work protectively. We also investigated the behavioral phenotype of mice with ~50% reduction in 14-3-3epsilon protein expression and found that these mice displayed weak phenotypes consistent with some aspects of human schizophrenia.

RESULTS

Screening analysis of *DISC1*-related genes and identification of *YWHAE* as a possible susceptibility gene for schizophrenia

To investigate whether novel DISC1-interacting molecules such as NUDEL, LIS1, YWAHE, GRB2 and KIF5A are associated with schizophrenia, we performed genetic association analyses using a Japanese population.

We failed to develop the genotyping of three SNPs in *LIS1* (*rs8082331*, *rs12938775* and *rs4790348*) and one SNP in *GRB2* (*rs16967795*), therefore a total of 25 SNPs were assessed in this analysis.

Though genotype distributions of two SNPs significantly deviated from Hardy–Weinberg Equilibrium (HWE, $P_{HWE} = .0143$: *rs4789172* in case sample, and $P_{HWE} = .0171$: *rs11172247* in control sample), those of the other markers were in HWE. Six tagging SNPs in *YWHAE* were significantly associated with schizophrenia and also *YWHAE* showed gene-wide significance (permutation $P = 0.0021$), whereas we found no association of tagging SNPs in *NUDEL*, *LIS1*, *GRB2* or *KIF5A* (Table 1).

Since six tagging SNPs in *YWHAE* located in the intron region, we performed denaturing high-performance liquid chromatography (dHPLC) analysis in 5′ flanking regions and entire exon regions of *YWHAE* to identify the possible causal polymorphism, and detected two SNPs: one in the 5′ flanking region (−261 bp from the initial exon: *rs28365859*) and the other one in the 3′-UTR (*rs9393*). Since the 5′ flanking region SNP might have a functional effect due to its position, we focused on this SNP in the following analysis [linkage disequilibrium (LD) structure of first-set samples in *YWHAE* can be seen in Fig. 1).

First, to examine the association of this SNP, we expanded the sample size (1065 cases and 1386 controls in a second set of confirmation samples, for a total of 1429 cases and 1728 controls including the first set of screening samples, call rates were 100%), and significant association was obtained ($P_{allele} = 1.01 \times 10^{-5}$ and $P_{genotype} = 4.08 \times 10^{-5}$). Furthermore, the significance could be detected in either set independently (Table 2). The commonly observed feature of these analyses was that the minor allele frequencies (MAFs) of this SNP were higher in controls than in schizophrenia patients. There was no discrepancy out of 380 randomly selected samples (190 cases and 190 controls) genotyped in duplicate and by another method (TaqMan Assay: C12125119) for this marker, suggesting it is unlikely that genotyping error had occurred.

Functional analysis of the promoter SNP in *YWHAE*: *in vitro* and *in vivo* expression assays

We first investigated the influence of *rs28365859* on *YWHAE* expression by dual-luciferase assay, although there is no evidence that the region where this SNP is located on is evolutionally conserved and that any regions in *YWHAE* are match as a core promoter by in silico promoter detection software. As shown in Fig. 2, a trend for significance in a promoterless vector and significance in a promoter vector were obtained in the different cell lines. The constructs containing a minor allele (C allele) showed higher expression in the promoter vector, suggesting that the C allele plays a possible enhancer role in these cell lines.

Next, to examine the role of this SNP in peripheral blood of healthy control subjects, real-time RT–PCR and western blot analysis were performed. Similar to the luciferase assays, heterozygous and homozygous minor allele (G/C and C/C genotype) subjects showed higher expression levels of 14-3-3epsilon than did homozygous major allele (G/G genotype) subjects (one-way analysis of variance, ANOVA, $P = 0.0251$ and 0.0014 in real-time RT–PCR and western blot analysis, respectively). Experimental analysis were performed to examine the differences under an additive model (G/G

Table 1. Screening analysis of DISC1-related genes

Gene	SNPs		Position[a]	Missing rate (%)	MAF Cases	Controls	*P*-value Allele	Genotype
NUDEL	rs3744652	C>T	8280008	0.3	33.0	35.9	0.250	0.274
	rs8064655	C>T	8301185	0	33.2	36.3	0.228	0.246
LIS1	rs1266474	A>G	2481460	0.4	9.72	12.4	0.110	0.0876
	rs4790356	G>A	2532979	0	10.6	11.7	0.528	0.730
	rs7212450	C>G	2538690	0	42.3	41.7	0.821	0.907
YWHAE	rs34041110	C>T	1193642	0	48.9	42.5	**0.0166**	**0.00563**
	rs9393	A>G	1195142	0	27.3	27.9	0.805	0.868
	rs8064578	C>T	1201625	0	48.5	43.4	0.0562	0.117
	rs7224258	G>C	1202252	2.1	15.0	20.3	**0.0102**	**0.0342**
	rs3752826	G>T	1211814	0	48.6	42.1	**0.0139**	**0.0175**
	rs7214541	T>C	1220072	0	44.6	49.4	0.0725	0.107
	rs11655548	A>G	1230748	2.3	29.3	38.3	**0.000418**	**0.00162**
	rs2131431	A>C	1241645	0.3	13.2	18.5	**0.00598**	**0.0176**
	rs1873827	A>G	1247690	0	42.4	49.6	**0.00732**	**0.0136**
	rs12452627	C>T	1249222	0	17.7	19.6	0.367	0.662
GRB2	rs7219	T>C	70826963	0	9.07	6.85	0.125	0.239
	rs8079197	C>G	70828274	0.6	8.45	6.60	0.190	0.308
	rs4789172	C>T	70853307	0.6	24.9[c]	26.1	0.617	0.659
	rs2053156	T>G	70890035	0	6.04	4.53	0.206	0.344
	rs930296	G>A	70915763	0	5.91	4.66	0.298	0.432
KIF5A	rs11172247	C>G	56232777	0	39.4	38.3[b]	0.676	0.609
	rs11172254	G>A	56255005	0.3	19.5	21.2	0.422	0.679
	rs775250	C>A	56263307	0	20.8	21.7	0.672	0.690
	rs775251	C>T	56265007	0.4	27.7	32.2	0.0713	0.129
	rs1678536	C>G	56265457	0.1	47.9	47.4	0.833	0.644

YWHAE showed gene-wide significance (permutation $P = 0.0021$).
Bold numbers represent significant *P*-values (<0.05).
[a]Based on HapMap database release#21.
[b]deviated from Hardy–Weinberg equilibrium.
MAF, minor allele frequency.

versus G/C+C/C), again significant associations were obtained.

Furthermore, haplotype trend regression test was applied to check the effects of haplotypes of *rs28365859* and other four SNPs in intron 1 (*rs11655548*, *rs2131431*, *rs1873827* and *rs12452627*), which might also be in an enhancer region. This showed significant association in either analysis ($P = 0.0282$ and 0.0186 in real-time RT–PCR and western blot analysis, respectively), however, each SNP in intron 1 was not correlated with the expression level (data not shown).

Effect of reduction of 14-3-3epsilon protein on the cognitive functions of mice

14-3-3 proteins are highly conserved across species, from bacteria to humans, and bind to phosphoserine/phosphothreonine motifs in a sequence-specific manner (24–28). Previously we reported that 14-3-3epsilon binds to CDK5/p35-phosphorylated NUDEL and maintains NUDEL phosphorylation. To examine the protective effect of 14-3-3epsilon on schizophrenia using mice, we should investigate whether overexpression of 14-3-3epsilon results in resistance for the onset of schizophrenic symptoms. However, an assay system to evaluate the effect of a gene on the onset of schizophrenia in mice has not yet been developed. Thus, in support of a role for *YWHAE* in schizophrenia, we investigated *Ywhae* knockout mice. Null mice of *Ywhae* gene ($Ywhae^{-/-}$) show a severe cell migration defect in both the cortex and the hippocampus, whereas $Ywhae^{+/-}$ mice, in which the expression level of 14-3-3epsilon protein is reduced to ~50% compared with their wild-type littermates, show a milder migration defect (29). Because most $Ywhae^{-/-}$ mice die at birth as previously reported (29), $Ywhae^{+/-}$ mice and their wild-type littermates were analyzed by a comprehensive behavioral test battery to investigate whether the reduction in 14-3-3epsilon protein affects behavior (30,31). $Ywhae^{+/-}$ mice appeared normal, healthy and fertile (Table 3).

To examine whether reduction in 14-3-3epsilon was associated with cognitive deficits, we analyzed $Ywhae^{+/-}$ mice and their wild-type littermates in working memory and reference memory tasks (Table 3). To assess working memory of $Ywhae^{+/-}$ mice, we used a spatial working memory version of the 8-arm radial maze task (32,33). The mice were trained for 26 trials. During training, both control and mutant mice improved their performance and no significant difference was observed ($P = 0.3325$) (Fig. 3A). The number of revisiting errors of $Ywhae^{+/-}$ mice was significantly more than their wild-type littermates during trials with a delay of 300 s ($P = 0.0229$) (Fig. 3C). The number of different arms chosen during the first eight choices, which is considered a measure of working memory that is relatively independent of locomotor activity levels and the total number of choices, was not significantly affected by the deficit of 14-3-3epsilon protein during training and trials with 30, 120 and 300 s of delay ($P = 0.3325$, 0.8972, 0.6476 and 0.5077, respectively) (Fig. 3B and D). These results

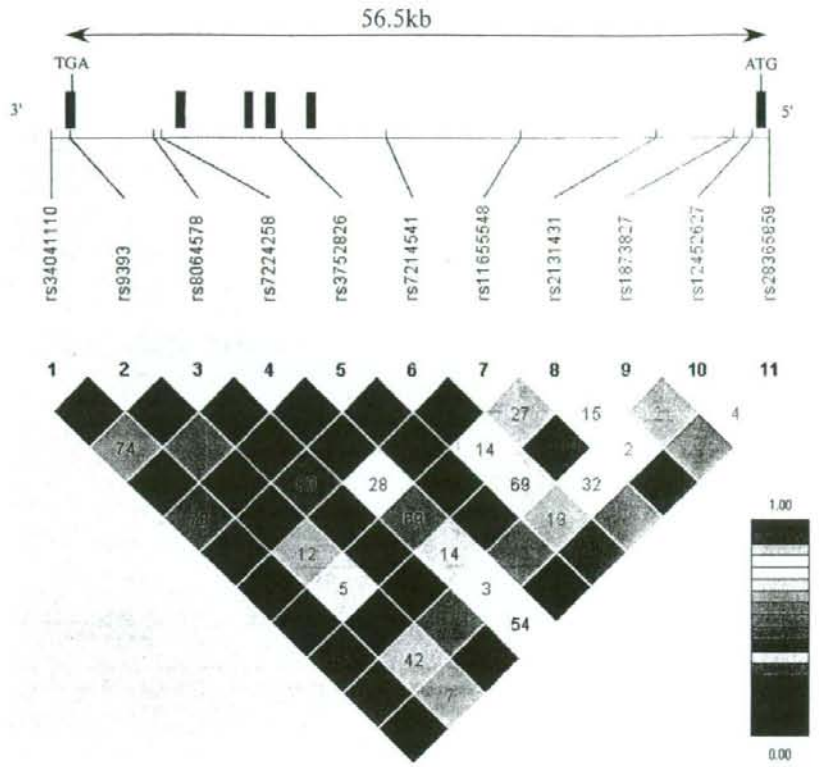

Figure 1. Tagging SNPs and LD evaluation of *YWHAE* for first-set screening samples. rs28365859 was included. Vertical bars represent exons. Numbers in boxes represent r^2 values, which should be expressed as decimals. r^2 values of 1.0 are not shown. Color scheme was based on GOLD format. Additional information is provided at the Haploview website.

Table 2. Association analysis of promoter SNP in YWHAE (rs28365859)

Samples[a]	Phenotype	*n*	Genotype G/G	G/C	C/C	MAF (%)	*P*-values HWE[b]	Allele	Genotype
Combined	Cases	1429	921	457	51	19.6	0.537	1.01×10^{-5}	4.08×10^{-5}
	Controls	1728	1000	620	108	24.2	0.366		
First-set	Cases	364	245	106	13	18.1	0.715	0.00108	0.00545
	Controls	342	192	127	23	25.3	0.748		
Second-set	Cases	1065	676	351	38	20.0	0.359	0.00123	0.00280
	Controls	1386	808	493	85	23.9	0.399		

First-set samples were identical to those used in screening analysis.
Second-set samples were independent set of samples to increase the sample size.
[a]Combined samples = first-set+second-set samples.
HWE, Hardy–Weinberg equilibrium.

suggest that *Ywhae*$^{+/-}$ mice show weak defects in working memory.

Next, we analyzed reference memory of *Ywhae*$^{+/-}$ mice, using the left–right discrimination test version of the T-maze. *Ywhae*$^{+/-}$ mice and their wild-type littermates were trained for 6 trials; then the correct side was reversed. The next 6 trials were performed under the reversal-learning condition. No significant difference was observed in the percentage of correct choices at the sixth trial (*Ywhae*$^{+/+}$, 80.647%; *Ywhae*$^{+/-}$, 77.157%; $P = 0.7516$), and no significant difference was observed under the reversal-learning condition ($P = 0.4567$) (Table 3). These results suggest that a decrease in the 14-3-3epsilon protein results in weak defects, specifically in spatial working memory.

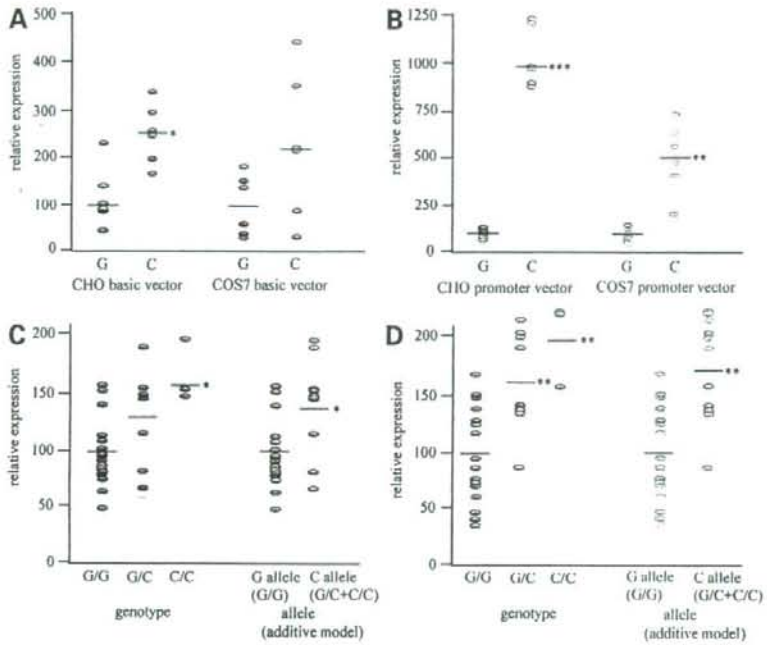

Figure 2. *In vitro* and *in vivo* expression assays. (**A**) Promoterless vector (basic vector) and (**B**) promoter vector in CHO and COS7 cells. Firefly luciferase activities were normalized with Renilla luciferase activities. Relative expression was calculated as 100 for the major allele (G allele) of *rs28365839*. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (in Student's *t*-test). Results of (**C**) real-time RT–PCR and (**D**) western blot analysis. Expression levels of 14-3-3epsilon RNA and protein were normalized with GAPDH expression. Relative expression was calculated as 100 for the major allele homozygous genotype (G/G genotype) or major allele (G allele) of *rs28365839*. Number of individuals with the distinct genotypes of *rs28365839* were 16 for G/G, 8 for G/C and 3 for C/C. $^{*}P < 0.05$, $^{**}P < 0.01$ (in *post hoc* comparison with the Dunnett test for genotype-wise analysis, and *t*-test for allele-wise analysis).

Moderately enhanced anxiety-like behavior in *Ywhae*$^{+/-}$ mice in the elevated plus-maze test

To examine the effect of 14-3-3epsilon deficit on anxiety-like behavior, *Ywhae*$^{+/-}$ mice and their wild-type littermates were analyzed in light/dark transition and elevated plus-maze tests. In light/dark transition, no significant difference was observed between *Ywhae*$^{+/-}$ mice and their wild-type littermates (Table 3). In the elevated plus-maze test, *Ywhae*$^{+/-}$ mice showed a smaller number of total entries ($P = 0.0075$) (Fig. 3E), increased time spent on closed arms ($P = 0.0195$) (Fig. 3F) and decreased time spent on center area ($P = 0.0012$) (Table 3). A significant difference was not observed in the number of entries onto open arms, total distance travelled or time spent on open arms ($P = 0.2044$, 0.1071, 0.3798, respectively) (Table 3). Thus, it is conceivable that *Ywhae*$^{+/-}$ mice have moderately enhanced anxiety-like behavior that could be detected only by the elevated plus-maze test but not by the light/dark transition or by the open-field tests.

DISCUSSION

Association between *YWHAE* and schizophrenia

In this study, we have identified *YWHAE*, the gene encoding 14-3-3epsilon, which forms a complex with DISC1 *in vivo*, as a possible susceptibility gene for schizophrenia. Genetic and expression evidence indicates that the SNP in 5′ flanking region (*rs28365859*) is associated with schizophrenia through influencing the expression level of *YWHAE*. Subjects with the C allele of *rs28365859* were thought to have a reduced risk of schizophrenia [odds ratio of combined subjects = 0.76 (95% confidence interval: 0.68-0.86)]. Our sample size was relatively large (3157samples consisting of 706 first-set and 2451 second-set samples: 1429 schizophrenics and 1728 controls), making our results reliable. In addition, another research assessing the genetic association of *YWHAE* with suicide victims [two of SNPs (*rs3752826* and *rs9393*) are identical SNPs in our study and another SNP (*rs1532976*) can be captured by *rs3752826* using HapMap information] can support our results, since it showed the same trends in the distributions of MAFs (MAFs of these SNPs were higher in controls than in cases) (34). However, a couple of limitations should be outlined. First, our results that show statistical significances may be derived from unknown population stratification, since Genomic Control was not included in this analysis. Secondly, there could be a possible effect of differential age distribution between cases and controls in the association analysis.

The *in vitro* luciferase assay suggests that the C allele might act as an enhancer, since significant luciferase induction could not be seen with the use of a promoterless vector, but luciferase activity (LA) could be assayed from the vector containing a promoter. Further, *in vivo* expression assays of RNA and

Table 3. Comprehensive behavioral test battery

Test		*Ywhae*$^{+/+}$	*Ywhae*$^{+/-}$	*P*-value	*F* value
General health					
Weight (g)		28.6	29.682	0.0262*	1.335
Rectal temperature (°C)		37.033	36.688	0.0435*	4.406
Pain test					
Hot plate (latency, s)		6.206	5.053	0.1142	2.633
Motor tests					
Grip strength (*n*)		1.044	1.085	0.2825	1.194
Wire hang (latency to fall, s)		60	50	0.0234*	5.65
Rotarod (latency to fall, s; average of six trials)		161.759	182.618	0.3391	0.941
Anxiety-like behavior					
Light/dark transition					
Distance travelled (cm)	Light side	484.983	617.782	0.0728	3.434
	Dark side	1095.389	1099.288	0.97	0.001
Stay time in light side (s)		214.972	231.176	0.6043	0.274
Transitions (times)		35.111	33.588	0.6827	0.17
Latency to light side (s)		31.444	34.941	0.6683	0.187
Elevated plus-maze					
Number of entries (times)		32.556	25.118	0.0075**	8.126
Entries onto open arms (%)		31.824	26.648	0.2044	1.677
Distance travelled (cm)		1323.722	1194.329	0.1071	2.744
Time on open arms (%)		15.269	12.971	0.3798	0.793
Time on closed arms (%)		50.87	63.196	0.0195*	6.034
Time on center area (%)		35.034	23.283	0.0012*	12.495
Depression model					
Porsolt forced swim (immobility, %)	Day 1	59.369	65.648	0.0661	3.614
	Day 2	77.026	78.564	0.6256	0.243
Tail suspension (immobility, %)		26.194	22.774	0.6267	0.241
Locomotor activity					
Open field					
Total distance travelled (cm)		8745.222	9258.941	0.5822	0.309
Vertical activity (times)		208.722	393.824	0.047*	4.259
Center time (s/min)		1.432	1.107	0.6505	0.209
Stereotypic counts (times)		7260.944	6124.118	0.2251	1.528
Sensory motor gating					
Acoustic startle response		3.021	2.704	0.32	1.02
Prepulse inhibition (startle stimulus, %)					
110-dB startle		48.887	47.697	0.8496	0.037
120-dB startle		13.566	16.6	0.5617	0.344
Working memory					
8-arm radial maze					
Training					
Different arm choice in first 8 entries (times)		6.209	6.016	0.3325	0.967
Revisiting errors (times)		6.12	7.613	0.1557	2.11
Delay 30 s					
Different arm choice in first 8 entries (times)		6.5	6.471	0.8972	0.017
Revisiting errors (times)		3.417	3.676	0.7599	0.095
Delay 120 s					
Different arm choice in first 8 entries (times)		6	5.882	0.6476	0.213
Revisiting errors (times)		4.944	6.735	0.1715	1.954
Delay 300 s					
Different arm choice in first 8 entries (times)		6.167	5.971	0.5077	0.448
Revisiting errors (times)		3.778	6.294	0.0229*	5.698
Reference memory					
T-maze (correct, %)	Training	80.648	77.157	0.0696	3.519
	Reverse	61.759	59.314	0.4567	0.567
Cued and contextual fear conditioning					
Conditioning (freezing, %)		28.324	29.29	0.7581	0.096
Context testing (freezing, %)		50.998	46.611	0.5754	0.32
Cued testing with altered context freezing, %)		53.641	52.926	0.8342	0.045
Social interaction					
Total duration of contact (s)		118.386	153.383	0.1239	2.776
Number of contacts (times)		49.429	53.333	0.4968	0.494
Total duration of active contacts (s)		14.257	18.733	0.0809	3.693
Mean duration/contact		2.443	3.017	0.2889	1.241
Distance travelled (cm)		2789.357	2882.167	0.7206	0.135

Behavioral test battery was performed in the following order: general health/neurological screen, wire hang, grip strength test, light/dark transition, open field, elevated plus-maze, hot plate, social interaction (novel environment), rotarod, prepulse inhibition, Porsolt forced swim, eight arm radial maze, T-maze, cued and contextual fear condition test, latent inhibition, tail suspension test.
*$P < 0.05$, **$P < 0.01$.

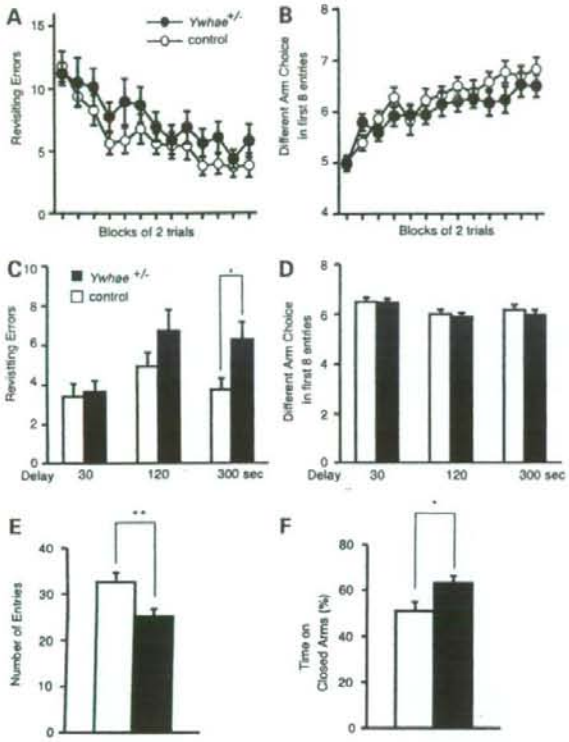

Figure 3. Behavioral abnormality of *Ywhae*$^{+/-}$ mice. (**A–D**) Working memory test of *Ywhae*$^{+/-}$ mice in the 8-arm radial maze (A and B). Total number of revisiting errors (A) and the number of different arms chosen in the first 8-arm visits (B) across training were counted. Data are presented as 2-day/trial averages. (C and D) Total number of revisiting errors (C) and the number of different arms chosen in the first 8-arm visits (D) of mice after training; exposure to delays of 30, 120 or 300 s after four pellets had been taken were counted (see *Materials and Methods*). (**E** and **F**) Anxiety-like behavior test of *Ywhae*$^{+/-}$ mice in the elevated plus-maze. (E) Number of total entries. (F) Time spent on closed arms. Number of total entries was lower and time spent on closed arms were greater in *Ywhae*$^{+/-}$ mice than in controls. Asterisks indicate a difference from the values of control mice. $^{*}P < 0.05$, $^{**}P < 0.01$.

protein in peripheral blood samples clarified the functional relevance of this SNP: Subjects who were heterozygous and homozygous with the C allele had higher expression of 14-3-3epsilon. Of note, our samples were control subjects not on medication; therefore, we could avoid the bias related to drug effects, which may be seen when studying schizophrenia subjects.

Also, haplotype trend regression analysis showed that the haplotypes consisted of five SNPs located in 5′ flanking region (*rs28365859*) and intron1 (*rs11655548*, *rs2131431*, *rs1873827* and *rs12452627*) were correlated with the expression level of *YWHAE*, whereas each SNP in intron 1 was not correlated with the expression. Therefore, we speculate that this significant result in haplotype-wise analysis may be derived mainly from the effects of *rs28365859*.

We analyzed for the homology of genome sequence between human and mice *Ywhae* gene using 500 bp upstream region from start ATG. About 200 bp upstream region from start ATG shows high identity, however, a region containing *rs2836589* SNP does not show homology. This result suggests that this SNP is not evolutionally conserved. We searched for functional motif on the sequence in the 5′ upstream region of *YWHAE* including *rs28365859* using TESS: Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess). In minor allele (C allele), ubiquitously expressed cellular upstream stimulatory factor (USF) -interacting motif 'CCACGT' was detected in this in silico analysis. This result may explain a possible functional effect of this SNP, an upregulation of 14-3-3epsilon in C allele-harboring people, however, further analysis would be needed to provide definitive conclusion.

Role of 14-3-3epsilon in neuronal development

Several observations of the postmortem brain suggest that alterations in neuronal cell migration, and synaptic, dendritic and axonal organizations occur in schizophrenia patients (35,36). *Ywhae*$^{+/-}$ mice show milder migration defects in both the cortex and the hippocampus, whereas *Ywhae*$^{-/-}$ mice display severe neuronal migration defects (29). Primary hippocampal neurons from *Ywhae*$^{-/-}$ mice display shorter axons and a defect in accumulation of the NUDEL/LIS1 complex in the distal part of axons (29). We confirmed that knockdown of 14-3-3epsilon by RNAi impairs not only the NUDEL/LIS1 complex transport into axons but also axon elongation (data not shown). Previously, we identified 14-3-3epsilon as an interacting molecule of *DISC1* (22). DISC1 is required to transport the NUDEL/LIS1/14-3-3epsilon complex into axons (22). Of note, depletion of endogenous DISC1 by RNAi results in a severe neuronal migration defect in the developing neocortex via regulation of the dynein complex (37). These results and reports suggest that both DISC1 and 14-3-3epsilon are required for neuronal development via transport of the NUDEL/LIS1 complex. To clarify the functional relationship between 14-3-3 epsilon and DISC1 on neuronal development via transport of the NUDEL/LIS1 complex, further genetic analysis using knockout mice will be required.

Cognitive dysfunction of *Ywhae*$^{+/-}$ mice

Ywhae$^{+/-}$ mice, in which the expression of 14-3-3epsilon protein was reduced to ~50% compared with their wild-type littermates, showed weak cognitive dysfunction specifically in working memory (Table 3). Interestingly, missense mutant mice of the *DISC1* gene show defects in working memory (38). Reduction of DISC1 or 14-3-3epsilon results in developmental defects of hippocampal neurons. These results and reports suggest that impairment of DISC1 or 14-3-3epsilon cause neuronal developmental defects, that result in cognitive dysfunction. Interestingly, impairment of working memory is one of the prominent features of schizophrenia symptomatology (39–41). Non-synonymous polymorphism of *DISC1* that consists of a serine to cysteine substitution at codon 704 (*DISC1*Ser704Cys) is reported to correlate with variations in hippocampal size and cognitive function including working memory, and is associated with

schizophrenia (42). Although relation between 14-3-3epsilon and cognitive function in human is not known, 14-3-3epsilon could be implicated in cognitive function that is associated with DISC1. Another prominent feature of schizophrenia symptomatology, prepulse inhibition (43), did not differ in *Ywhae*$^{+/-}$ mice compared with their wild-type littermates (Table 3). Schizophrenia is a complex disorder with a variety of pathology and risk factor genes. It is a reasonable assumption that modification of a single gene does not mimic all features of schizophrenia symptomatology. We think that our results using *Ywhae*$^{+/-}$ mice partly support our genetic data. However, further analysis would be required to clarify a role of 14-3-3epsilon on cognitive functions and functional relationship between *YWHAE* and *DISC1*.

YWHAE as a possible susceptibility gene for schizophrenia

In this study, we found that a SNP of *YWHAE* that correlates the expression of 14-3-3epsilon is associated with schizophrenia, and that this SNP would reduce the risk of schizophrenia. Perhaps, increased 14-3-3epsilon expression in humans affected by the identified SNP is protective, whereas decreased 14-3-3epsilon expression due to 50% reduction by heterozygous knockout in mice results in behavioral deficits. At this point, we do not know why higher expression levels of 14-3-3epsilon reduce the risk of schizophrenia, or why lower expression levels of this gene result in increase of the risk in human and behavioral changes in mice. By its susceptibility genes, schizophrenia seems to be a complex disorder with multiple symptoms and genetic risk factors. We predict that schizophrenia would be divided into several classes by its susceptibility genes. Each class would have its own molecular/signaling pathway that plays important roles in the pathogenesis. DISC1 and its interacting molecules are required in neuronal developments and adult neurongenesis (44), and would play critical roles in pathogenesis of specific classes of schizophrenia. In other classes of schizophrenia, the DISC1-pathway would not be implicated in the pathogenesis. Some genes could have redundant functions. 14-3-3epsilon is a member of adaptor proteins that interact with phosphorylated serine or threonine residue of target proteins. More than 100 of 14-3-3-binding partners involved in signal transduction, cell cycle regulation, apoptosis, stress responses and malignant transformation have been identified (45). Proteomic analysis of synapse revealed that 14-3-3epsilon forms a complex with NMDA receptor (46). Placing these results and reports in the context of the pathogenesis of schizophrenia, 14-3-3epsilon could be a susceptibility gene of not only DISC1-implicated, but also wide range of schizophrenia because of its wide variety of interacting partners. 14-3-3epsilon would be a key molecule to understand molecular mechanisms of susceptibility genes for schizophrenia.

MATERIALS AND METHODS

Subjects in genetic association analyses

In the genetic association analyses, two independent sets of subjects were examined. The first screening analysis included 364 patients with schizophrenia (188 male and 176 female; mean age $\pm$ SD 42.5 $\pm$ 14.8 years) and 342 healthy controls (191 male and 151 female; 35.0 $\pm$ 13.6 years). Patients for the second confirmation analysis included 1065 patients with schizophrenia (562 male and 503 female; 48.9 $\pm$ 14.7 years) and 1386 controls (714 male and 672 female; 42.6 $\pm$ 14.6 years). All subjects were unrelated to each other and reported to be of Japanese ethnicity. Forty patients with schizophrenia were used as subjects for a mutation search; these subjects were also included in the first-set screening scan. The schizophrenia patients were diagnosed according to criteria in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* after at least two experienced psychiatrists reached consensus on the diagnosis on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened on the basis of unstructured interviews; to exclude subjects with any brain disorder, or psychotic disorder, or who had first-degree relatives with psychotic disorders, trained psychiatrist interviewed them to assess current and/or past mental states (psychotic, mood, anxiety, obsessive-compulsive symptoms) and family history. After description of the study, written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University, Teikyo University, Okayama University, and Nagoya University Graduate School of Medicine.

SNP selection and genotyping

For LD-based association analysis using the first set of screening samples, we first consulted the HapMap and dbSNP databases to pick-up 'tagging SNPs'. From the HapMap database (Data Release #21: population JPT: MAF of >0.05: regions 8275000.8320000 for NUDEL, 2440000.2537000 for *LIS1*, 1193000.1256000 for *YWHAE*, 70823000.70917000 for *GRB2*, 56227000.56266000 for *KIF5A*), we selected a total of 27 tagging SNPs (one SNP for *NUDEL*, six SNPs for *LIS1*, nine SNPs for *YWHAE*, six SNPs for *GRB2* and five SNPs for *KIF5A*) with a threshold criterion of $r^2 > 0.8$ in pairwise tagging mode using Tagger software (47). Two SNPs (one for *NUDEL*, rs3744652 and one for *YWHAE*, rs34041110) were added for denser mapping.

All SNPs were genotyped by TaqMan assays, primer extension using dHPLC and polymerase chain reaction–restriction fragment length polymorphism assays as described previously (48). More detailed assay information can be found in Supplementary Material, Table S1.

Mutation search

After we detected significant association of *YWHAE* in screening samples, we used dHPLC analysis for a mutation search, the details of which are described in a previous paper (48). Primer pairs (Supplementary Material, Table S2) were designed with the use of information from the GenBank sequence (accession number: NT 010718.15) into 10 amplified regions, which covered all the coding regions, the branch sites and the 5′ flanking region 1026 bp upstream from the initial exon of *YWHAE*.

In vivo and *in vitro* expression assays

We used a dual-luciferase assay, real-time RT–PCR and western blot analysis to examine the influence of SNP *rs28365859* in the 5′ flanking region on expression levels of *YWHAE*. For the dual-luciferase assay, 497-bp fragments that included *rs28365859* were PCR amplified (Supplementary Material, Table S1). Genomic DNAs with identified genotypes were used as templates, and PCR products of either genotypes were cloned into a pGL3-basic vector and a pGL3-promoter vector (Promega, WI). These vectors with both alleles, the Renilla luciferase vector and the phRL-TK vector, were transiently transfected into Chinese hamster ovary (CHO) cells and COS-7 cells with the use of Lipofectamine 2000™ (Invitrogen, CA). All inserts were sequenced to confirm the containing alleles. After 48 h, cell extracts were prepared and assayed for firefly LA (LA_F) and Renilla LA (LA_R) as described by the manufacturer (Pikka-Gene Dual SeaPansy™ Luminescence Kit, Tokyo Ink, Japan) on a Fluoroskan Ascent FL (Thermo Labsystems, Finland).

For *in vitro* assays (real-time RT–PCR and western blot analysis), we processed and analyzed a total of 27 peripheral blood samples from normal control subjects to determine the amount of *YWHAE* transcript or protein: 16 subjects with homozygous major alleles (G/G genotype: 7 male and 9 female; 32.6 ± 6.4 years) in *rs28365859*; 8 subjects with heterozygous major alleles (G/C genotype: 4 male and 4 female; 33.5 ± 7.7 years) and 3 subjects with homozygous minor alleles (C/C genotype: 1 male and 2 female; 51.3 ± 17.0 years). These subjects were healthy controls who had not received any medication within at least 1 month before the collection of RNA and protein.

In the real-time RT–PCR assay, total RNA was isolated with the use of a QIAamp RNA Blood Mini Kit (QIAGEN, Inc., CA). Complementary DNA was generated with the use of a High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR constituents were 50 ng DNA, 2× TaqMan Universal Master Mix and 20× primer/probe mix (Hs00356749_g1, Applied Biosystems) in a 50-μl final volume. The amplification was done according to the manufacturer's instructions, and signals were recorded during PCR with the use of an ABI PRISM 7900 instrument. All gene expression results were normalized to the expression of glyceryl aldehyde-3-phosphate dehydrogenase (*GAPDH*).

In the western blot analysis, lymphocytes were purified (Axis-Shield, Oslo, Norway) and protein concentrations were determined with bovine serum albumin as the reference protein. The antibody against 14-3-3epsilon and alpha-tubulin were purchased (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Proteins were subjected to SDS–PAGE, followed by immunoblot analyses with anti-14-3-3epsilon or anti-alpha-tubulin antibody. The amount of 14-3-3epsilon was detected by chemiluminescence in a linear range using serial dilutions of standards and was estimated with Densitograph (ATTO, Tokyo, Japan). Alpha-tubulin was used as the standard for quantification. The results of these *in vivo* and *in vitro* expression assays were representative of three independent experiments.

Animals and experimental design

Ywhae$^{+/-}$ mice and their wild-type littermates were obtained as previously reported (29). Genetic background of mice is mixed 129/S6 × NIH Black Swiss. All behavioral tests (8-arm radial maze test, elevated plus-maze test, T-maze test, light/dark transition test and startle response/prepulse inhibition tests) were carried out with male mice that were 9–10 weeks old at the start of the testing. Heterozygous knockout mice and wild-type littermates were compared in experiments. Mice were housed in a room with a 12-h light/dark cycle (lights on at 7:00 a.m.) with access to food and water ad libitum. Behavioral testing was performed between 9:00 a.m. and 6:00 p.m. After the tests, all apparatus was cleaned with super hypochlorous water to prevent a bias on the basis of olfactory cues with the apparatus. Detailed description of each behavioral test (neurological screen, neuromascular strength, rotarod test, open-field test, light/dark transition test, elevated plus-maze test, hot plate test, startle response/prepulse inhibition tests, social interaction test in a novel environment, sociability and social novelty preference test, social interaction test in home cage, T-maze test and contextual and cued fear conditioning) can be seen in Supplementary methods.

Statistical analysis

Tests for HWE and marker-trait association were evaluated by χ^2 test (SAS/Genetics, release 8.2, SAS Institute Japan Inc., Tokyo, Japan). Gene-wide significance of single-SNP test was estimated by permuting phenotype status to generate 10 000 data set of SNPs in each gene under null hypothesis of no association (49). Differences in relative expression between alleles (for luciferase assay) and genotypes (for real-time PCR and western blot) were evaluated by a two-tailed Student's *t*-test and one-way ANOVA, respectively (JMP5.1J, SAS Institute Japan Inc.). When a significant difference was obtained in ANOVA, *post hoc* comparison with the Dunnett test [with homozygous major alleles (G/G genotype) set as controls] was used to identify specific group differences. Also to check the effects of haplotypes on gene expression, haplotype trend regression test with permutation (10 000 times) was applied (Power Marker V3.25 by Jack Liu, www://power-marker.net/). In behavior analysis, statistical analysis was conducted by using STATVIEW (SAS Institute, Cary, NC). Data were analyzed by ANOVA or repeated-measures ANOVA. Values in graphs were expressed as mean ± SEM. The level of significance was set at 0.05.

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SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* Online.

Conflict of Interest statement: None declared.

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The anthraquinone derivative Emodin ameliorates neurobehavioral deficits of a rodent model for schizophrenia

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Summary. Abnormality in cytokine signaling is implicated in the neuropathology of schizophrenia. Previously, we established an animal model for schizophrenia by administering epidermal growth factor (EGF) to neonatal rats. Here we investigated effects of the anthraquinone derivatives emodin (3-methyl-1,6,8-trihydroxyanthraquinone) and sennoside (bis-[D-glucopyranosyl-oxy]-tetrahydro-4,4′-dihydroxy-dioxo[bianthracene]-2,2′-dicarboxylic acid) on behaviors of this model and EGF signaling. Subchronic oral administration of emodin (50 mg/kg) suppressed acoustic startle responses and abolished prepulse inhibition (PPI) deficits in this rodent model. ANCOVA revealed that emodin had distinct effects on PPI and startle responses. In contrast, sennoside (50 mg/kg) had no effects. Emodin attenuated weight gain initially during treatment but had no apparent effect on weight gain and locomotor activity thereafter. Application of emodin to neocortical cultures attenuated the phosphorylation of ErbB1 and ErbB2. We conclude that emodin can both attenuate EGF receptor signaling and ameliorate behavioral deficits. Therefore, emodin might be a novel class of a pro-drug for anti-psychotic medication.

Keywords: Antipsychotic; behavior; inflammation; ErbB; EGF; schizophrenia

Introduction

Emodin is an anthraquinone derivative, 3-methyl-1,6,8-trihydroxyanthraquinone, that is extracted and purified from rhubarb. This natural compound has been proposed to possess a variety of pharmacological activities including anti-inflammatory, antiviral, hepatoprotective and antiulcerogenic activities (Wang et al. 2001; Huang et al. 2007). Recent molecular studies indicate that this compound attenuates signal transduction of growth factors and cytokines, inhibiting ErbB2, src-family kinases, IkappaB kinase and MAP kinase (Jayasuriya et al. 1992; Kumar et al. 1998; Zhang et al. 1998, 1999; Wang et al. 2001, 2006, 2007; Li et al. 2005; Kaneshiro et al. 2006). Accordingly, emodin has been reported to exhibit anti-tumor activity against adenocarcinomas, leukemias and lung carcinomas (Lee 2003; Su et al. 2005; Muto et al. 2007). Despite the intensive study of emodin in tumor biology, the effects of this compound on the brain or behavioral traits are largely unknown (Gu et al. 2005; Lu et al. 2007).

The EGF receptors, ErbB1, are enriched in midbrain dopaminergic neurons (Seroogy et al. 1994). Abnormal expression or function of ErbB1 and ErbB2 has been implicated in Parkinson's disease and schizophrenia (Futamura et al. 2003; Mizuno et al. 2004; Iwakura et al. 2005; Tohmi et al. 2005). EGF signaling appears to be perturbed in patients with schizophrenia as ErbB1 levels are increased in the striatum of schizophrenia patients (Futamura et al. 2002). Genetic linkage studies also support the contribution of EGF signaling to the etiology and pathology of schizophrenia (Anttila et al. 2004; Hanninen et al. 2007). To study the mechanisms that contribute to the emergence and symptoms of schizophrenia, we established an animal model for schizophrenia by treating neonatal rats with subchronic doses of EGF. Treated rats later exhibit behavioral deficits in prepulse inhibition, social interaction, and exploratory locomotor activity. Some of these behavioral deficits are sensitive to antipsychotic medication (Futamura et al. 2003; Mizuno et al. 2004; Tohmi et al. 2005). In addition, subchronic exposure of neonates to EGF appears to permanently sensitize ErbB1 signaling (Nawa and Mizuno 2006). Thus, as emodin attenuates EGF receptor signal-

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ing, it may also ameliorate these associated behavioral deficits.

In this study, we investigated the effects of emodin and its derivative on the behavioral deficits associated with this EGF model for schizophrenia. Bearing in mind future therapeutic applications, these anthraquinone agents were given orally and the effects on startle responses and prepulse inhibition were examined at acute and/or subchronic phases of drug administration. In parallel, the side effects of emodin administration on weight gain and locomotion were evaluated. In addition, we examined the activity of emodin on EGF receptor signaling in primary neuronal cultures in order to correlate the molecular activity of emodin with its influence on behavior.

Materials and methods

Subjects

Neonatal Sprague-Dawley rats (postnatal day 2, 10 pups/L) were purchased with dams from SLC Co. Ltd (Shizuoka, Japan). Recombinant human EGF (Higeta Syoyu, Chiba, Japan) was dissolved in saline and subcutaneously administered to half of individual litters daily during postnatal day (PND) 2–10 at the nape of the neck at a dose of 0.875 mg/kg of body weight (Futamura et al. 2003). Control littermates received 0.875 mg/kg of cytochrome c (Sigma Chemical Co., St. Louis, MO, USA), and served as controls for all analyses. After PND20, rats were separated according to gender and raised separately (2–3 rats per cage; 25 L × 38W × 18H cm). All rats were maintained under a 12-h light-dark cycle (7:00 on −19:00 off) with free access to food and water.

Schedule of behavioral testing, drug treatment, and dissection

The cytochrome c- or EGF-treated rats were given emodin (5–50 mg/kg; 96–99% pure; Tokyo Chemical Industry Inc., Tokyo, Japan or 90% pure; Sigma Chemical Co.) or sennoside A (50 mg/kg; >90% pure, Wako Chemical Co., Osaka, Japan) as adults (PND56-62). Emodin or sennoside A was sonicated in a 10% lecithin solution (Wako Chemical Co.) at a final concentration of 5 mg/ml. This emulsion of emodin, sennoside, or vehicle (10% lecithin) was administered to rats once a day for 7 days with the aid of an oral zonde for rats (Natume Seisakusho Co. Ltd., Japan). One day after the last administration, rats were subjected to behavioral tests (see below). Alternatively, rats were given emodin orally (50 mg/kg) once and subjected to behavioral tests 3 h later. The given doses were set below the reported toxic amount of emodin (< 80 mg/kg) according to Jahnke et al. (2004). To minimize interactions between independent tests in Figs. 2, 3, and 5, rats were weighed, tested for locomotor activity and then tested for acoustic startle response. In the other experiments, rats were subjected to only one of the schedule tests. Behavioral tests were performed during the day cycle. In total, 6 experimental groups representing 130 rats were used in the present study. 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.

Rat neocortical culture

Whole cerebral neocortices of embryonic rats (Sprague-Dawley, embryonic day 18–19) were treated with papain solution (10 mg/ml; Sigma Chemicals), mechanically dissociated, and seeded onto poly-D-lysine-coated dishes at a density of 500 cells/mm^2 (Takei et al. 2004). Cortical neurons were maintained in Dulbecco's modified Eagle's medium containing 1 mM glutamine and 10% fetal bovine serum. After 6 days, neuronal cultures were pretreated with emodin (0–300 μM; Tokyo Chemical Industry) or sennoside A (0–300 μM; Wako Chemical Co.) for 2 h and challenged with EGF (5 ng/ml; Higeta Syoyu) for 5 min.

Immunoblot analysis

Polyacrylamide electrophoresis (PAGE) and immunoblotting were performed as described previously (Takei et al. 2004). Cells were harvested, lysed, and sonicated in sample buffer (10 mM Tris–HCl, 150 mM NaCl, 2% SDS, 20 mM NaF, 1 mM Na_3VO_4). After centrifugation, supernatant was collected and the protein concentrations were determined. Equal amounts of protein were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were probed with anti-phosphorylated-ErbB1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phosphorylated-ErbB2 (1:1000, Upstate, Lake Placid, NY, USA), anti-beta-actin antibodies (1:10000, Chemicon Int, Temecula, CA, USA), followed by horseradish-peroxidase-conjugated anti-mouse IgG or horseradish-peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:10000, DAKO Cytomation, Glostrup, Denmark). Peroxidase activity was visualized with chemiluminescence reaction (Western Lightning, Perkin Elmer, Tokyo, Japan) coupled with film exposure.

Measurement of acoustic startle and prepulse inhibition (PPI)

Acoustic startle and prepulse inhibition (PPI) responses were measured in a startle chamber (SR-Lab Systems, San Diego Instruments, San Diego, CA, USA) adapted for rats (Swerdlow and Geyer 1998; Swerdlow et al. 2001). This paradigm was used to assess startle amplitude, habituation and PPI response with acoustic stimuli of 120 dB, a single prepulse interval (100 msec), and three 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: (i) a 40-msec 120-dB noise burst presented alone (S); (ii–iv) a 40-msec 120-dB noise burst 100 msec after a prepulse (20-msec noise burst) at either 5, 10, or 15 dB above background noise (i.e., 75-, 80-, or 85-dB prepulse, respectively); or (v) no stimulus (N; background noise alone). The last condition was used to measure baseline movement in the chamber. In PPI test, these 5 trial types (i–v) were each repeated 8 times in a pseudorandom order, resulting in 40 total 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 (i) were presented to assess habituation during the session. The inter-trial interval was 15 sec. Analysis of PPI was based on the mean of the eight trials for each trial type. The percentage PPI of the startle response was calculated as:

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

Locomotor activity

We measured locomotor activity in a novel environment using a large size of behavioral chamber as described previously (Futamura et al. 2003). Each rat was placed in an open field box (45 cm length × 45 cm width × 30 cm height, MED Associates, St. Albans, VA, USA) under a moderate light level (400 Lx). Line crossings and rearing counts were measured by photo-beam sensors (25 mm intervals for horizontal axis and 150 mm for vertical axis) for 60 min.

Statistical analysis

Results are expressed as means ± SEM. Statistical differences were determined by analysis of variance (ANOVA) as well as by analysis of covariance (ANCOVA). 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 treatment (two levels), emodin administration (two or four levels) as a between-subject factors and prepulse magnitude (three levels) as a within-subject factor. Interaction of a within-subject factor with between-subject factors was estimated by ANCOVA. When the initial analyses yielded significant factorial interaction, the data were separated to avoid the interaction for the final analyses. Subsequently, a Fisher's LSD test was applied to absolute behavioral values as a *post hoc* test of multiple comparisons. A P value less than 0.05 was regarded as statistically significant. Statistical analysis was performed using the SPSS software (version 11.5). N values in parentheses represent the number of animals used.

Results

Effects of emodin oral administration on startle response and prepulse inhibition

Neurobehavioral impairments were induced in rats with the inflammatory cytokine EGF as described previously (Futamura et al. 2003; Mizuno et al. 2004; Tohmi et al. 2005). EGF or cytochrome c (a control compound for EGF) was administered daily to littermates of neonatal rats (PND2, $n=5$ for each group) for 9 days. At 8 weeks postnatal ($n=5$ each), vehicle or various doses of emodin (5, 15 and 50 mg/kg/day) were given daily (p.o.) to rats for 7 days. One day after the last emodin administration, startle responses to 120-dB tone and prepulse inhibition with 85-dB tones were monitored to estimate the effective dose of emodin. Two-way ANOVA with a between subject factor of neonatal EGF treatment (EGF and cytochrome c) and emodin dose (4 levels) revealed that neonatal EGF treatment exhibited a significant main effect on startle response [$F(1,32)=9.65$, $P=0.039$]. The main effect of emodin dose was not significant [$F(3,32)=0.91$, $P=0.45$] without interaction [$F(3,32)=0.272$, $P=0.85$] (Fig. 1A). In contrast, PPI levels were significantly increased by emodin administration in a dose-dependent manner. Repeated ANOVA with between subject factors of treatment (EGF and cytochrome c) and emodin dose (0, 5, 15, and 50 mg/kg/day) (Fig. 1B) revealed that there were significant main effects of EGF treatment [$F(1,32)=40.8$, $P<0.001$] and emodin dose [$F(3,32)=4.98$, $P=0.006$] with a significant interaction between EGF treatment and emodin dose [$F(3,32)=3.82$, $P=0.019$]. *Post-hoc* analysis indicated that the 15 and 50 mg/kg dose of emodin significantly elevated PPI levels of EGF-treated rats in comparison to the levels of EGF-treated rats not receiving emodin. In contrast, cytochrome c-treated group (control) failed to react with emodin. Accordingly, we detected significant differences between EGF-treated and cytochrome c-treated (control) groups at the doses of 0 and 5 mg/kg emodin, but not at higher doses. However, there were no

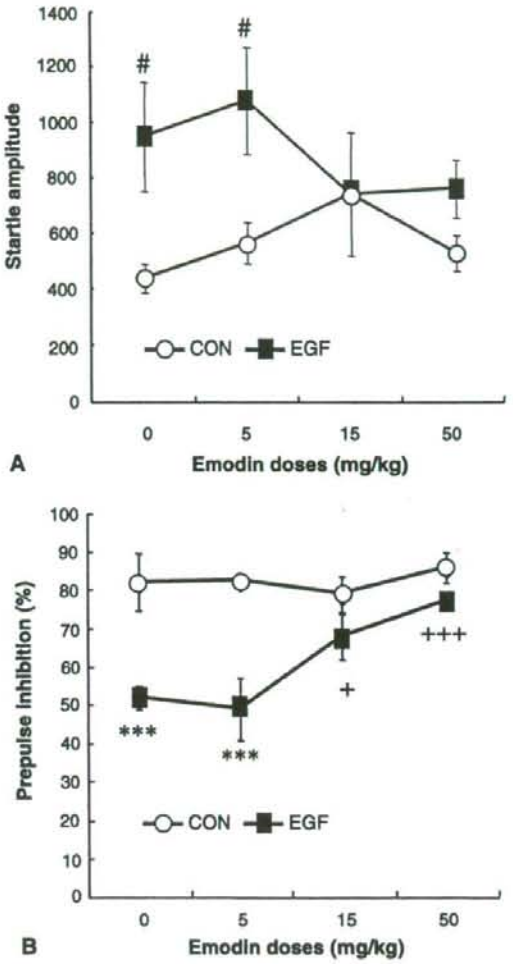

Fig. 1. Emodin dose dependency of PPI recovery in the EGF model. Different doses of emodin (0, 5, 15, and 50 mg/kg/day) were orally administered for 7 days to adult male rats that had been treated with EGF (*closed square*) or cytochrome c (*open circle*) as neonates. **(A)** The magnitude of pulse-alone startle (120 dB) was plotted against individual emodin doses. Values indicate means ± SEM ($n=5$ each). **(B)** Prepulse inhibition (PPI) with an 85 dB prepulse stimuli was measured and compared between doses. $^{***}P<0.001$, compared with cytochrome c-treated controls, and $^{+}P<0.05$, $^{+++}P<0.001$, compared with the EGF-treated group not receiving emodin, both by Fisher LSD. $^{\#}$The startle difference between EGF-treated and cytochrome c-treated groups were marginal at the zero and 5 mg/kg doses of emodin (both $P=0.057$) by Fisher LSD

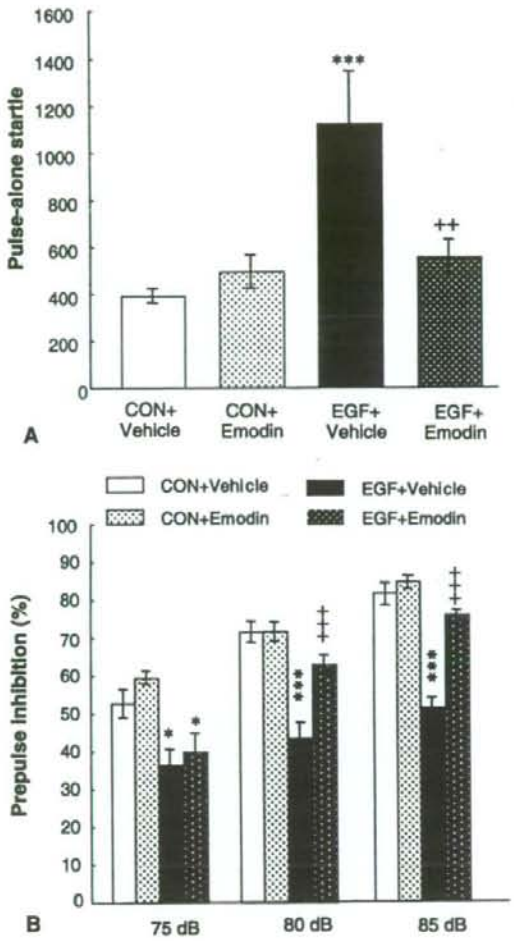

Fig. 2. Effects of subchronic emodin administration on the PPI deficits and startle responses of rats receiving EGF as neonates. Adult male rats were given an emodin emulsion or vehicle orally (10% lecithin) daily for 7 days. One day after the last dose of emodin, pulse-alone startle response to a 120-dB tone (**A**) and the percentage PPI with 75, 80, and 85 dB prepulse stimuli (**B**) were measured. Open and black bars represent cytochrome c-treated controls (*CON*) and EGF-treated rats (*EGF*) that received vehicle orally. Black dotted and white dotted bars represent cytochrome c-treated controls and EGF-treated rats that received emodin orally. Bar indicates mean ± SEM for each prepulse intensity ($n = 14$ each). $^{*}P < 0.05$, $^{***}P < 0.001$, compared with cytochrome c-treated controls, and $^{++}P < 0.01$, $^{+++}P < 0.001$, compared with EGF-treated controls that did not receive emodin at the same prepulse intensity, both by Fisher LSD

significant effects on PPI for other prepulse intensities (data not shown).

To confirm the results of this preliminary dose response study for emodin, we prepared four larger groups of animals that had been treated with EGF or cytochrome c as neonates and given either vehicle or emodin subchronically (50 mg/kg) as adults ($n = 14$ for each group) (Fig. 2). Pulse-alone startle responses (120 dB) were significantly altered by neonatal EGF treatment [$F(1,52) = 11.1$, $P = 0.016$, ANOVA] and marginally by emodin administration [$F(1,52) = 3.77$, $P = 0.058$, ANOVA] with a significant interaction between EGF treatment and emodin administration [$F(1,52) = 7.87$, $P = 0.007$, ANOVA] (Fig. 2A). *Post-hoc* analysis revealed that emodin administration specifically suppressed the pulse-alone startle increased by neonatal EGF treatment.

Emodin ameliorated the abnormality in prepulse inhibition (Fig. 2B). Three-way ANOVA with between subject factors of treatment (EGF and cytochrome c) and emodin administration (emodin and vehicle) and a within subject factor of prepulse intensities (75, 80, and 85 dB) revealed significant main effects of EGF treatment [$F(1,52) = 63.1$, $P < 0.001$] and emodin administration [$F(1,51) = 16.6$, $P < 0.001$], and a significant interaction between EGF treatment and emodin administration [$F(2,104) = 7.13$, $P = 0.010$]. We interpret from these data that emodin had a differential effect on EGF-treated and cytochrome c-treated animals. The effects of emodin were separately analyzed in either the EGF-treated or the cytochrome c-treated group. In the EGF-treated group, there was a significant effect of emodin administration on PPI [$F(1,26) = 17.5$, $P < 0.001$, repeated ANOVA] with an emodin × prepulse interaction [$F(2,52) = 9.86$, $P < 0.001$]. *Post-hoc* analysis detected significant effects of emodin administration for 80- and 85-dB prepulses. In contrast, emodin did not have an effect on PPI in the cytochrome c-treated group [$F(1,26) = 1.40$, $P = 0.25$, repeated ANOVA].

As emodin administration affected the pulse-alone startle and specifically PPI levels for higher prepulse stimuli, interpreting these results required detailed analysis (Swerdlow et al. 2001). To test the possibility that the increase in pulse-alone startle responses might promote the decrease in PPI, individual data for EGF-treated rats were re-analyzed by the Peason's correlation analysis followed by ANCOVA (Cadenhead et al. 1993) (Fig. 3). When the percent PPI levels for 85-dB prepulse were plotted versus the magnitude of the pulse-alone startle for each animal of the EGF-treated group, there was weak correlation between these values in vehicle-given rats ($R = -0.60$, $P = 0.023$ for vehicle and $R = +0.185$, $P = 0.54$ for emodin). This suggests that there was a weak contribution of the increase in pulse-alone startle to the reduction in PPI. As there was no significant difference in slope of the line for the vehicle and EGF-treated groups [$F(3,24) = 1.99$, $P = 0.17$], therefore, we re-valuated pure effects of emodin on PPI while assum-

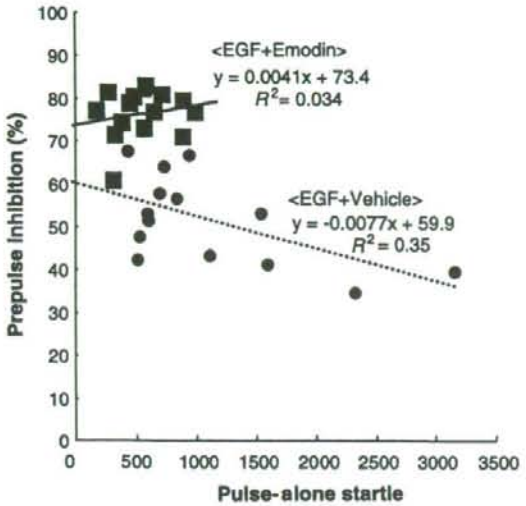

Fig. 3. The effects of emodin on the correlation between PPI and pulse-alone startle. Percentage of PPI obtained with an 85 dB prepulse was plotted against the pulse-alone startle for rats treated with EGF as neonates. The data correspond to Fig. 2. *Circles* represent PPI levels of individual EGF-treated rats given vehicle orally for one week and squares represent those of EGF-treated rats receiving emodin. Emodin administration did not significantly change the slope of the regression curves

ing that the pulse-alone startle influenced the levels of PPI. ANCOVA with the pulse-alone value as a covariate revealed that the group main effect of EGF treatment on absolute PPI retained significance [$F(1,24) = 42.2$, $P < 0.001$, ANCOVA]. Similar statistical results were obtained for the data obtained with an 80-dB prepulse [$F(1,25) = 10.1$, $P = 0.003$]. Thus, we conclude that emodin ameliorates the PPI deficits irrespective of its effect on pulse-alone startle.

Acute effects of emodin on startle response and prepulse inhibition

We also examined the immediate effect of emodin administration on prepulse inhibition (Fig. 4). Vehicle or emodin (50 mg/kg) was orally given to rats. Three hours after administration, startle responses were monitored in the presence and absence of the prepulse stimuli and PPI levels were calculated. Two-way ANOVA for pulse-alone startle revealed that there were no significant effects of emodin administration [$F(1,16) = 0.113$, $P = 0.74$] without interaction [$F(1,16) = 2.07$, $P = 0.17$] (Fig. 4A). Acute administration of emodin failed to mimic the subchronic effects. There was no significant main effect of emodin administration [$F(1,16) = 0.070$, $P = 0.79$] or interaction [$F(1,16) = 1.29$, $P = 0.28$] (Fig. 4B). The present result suggests that repeated administration of emodin is required to improve the PPI deficit induced by neonatal EGF treatment.

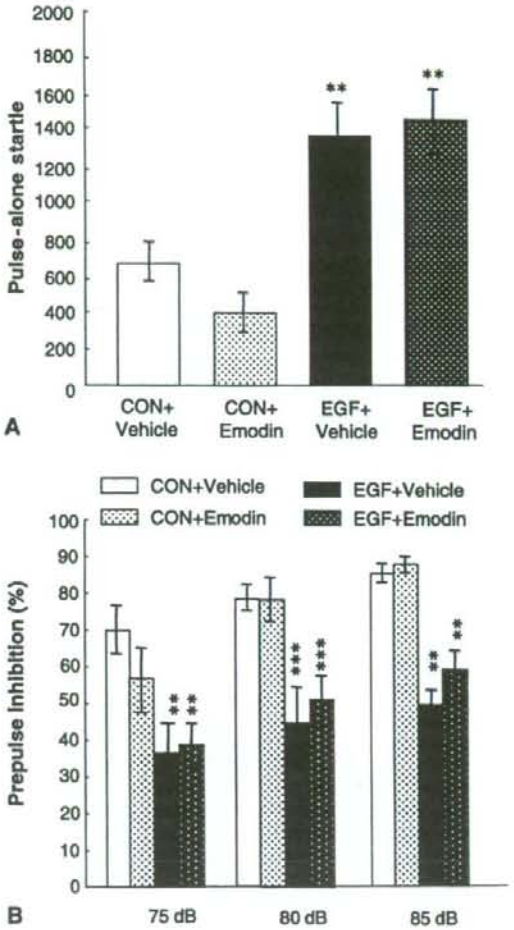

Fig. 4. Acute effects of emodin administration on pulse-alone startle and PPI in the EGF model. Adult male rats were given emodin emulsion or vehicle (10% lecithin) orally. Three hours after administration, pulse-alone startle response to a 120 dB tone (**A**) and percentage PPI with 75, 80 and 85 dB prepulse stimuli (**B**) was measured. Open and black bars represent cytochrome c-treated controls (*CON*) and EGF-treated rats (*EGF*) that received vehicle orally. Black dotted and white dotted bars represent cytochrome c-treated controls and EGF-treated rats that received emodin orally. *Bar* indicates mean ± SEM for each prepulse intensity ($n = 14$ each). $^{**}P < 0.01$, $^{***}P < 0.001$, compared with cytochrome c-treated controls at the same prepulse intensity by Fisher LSD

Side effects of subchronic emodin administration

Anthraquinone derivatives such as emodin are used as mild purgatives (Nakajima et al. 1985). We examined whether