The evolutionarily conserved G protein-coupled receptor SREB2/GPR85 influences brain size, behavior, and vulnerability to schizophrenia

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The G protein-coupled receptor (GPCR) family is highly diversified and involved in many forms of information processing. SREB2 (GPR85) is the most conserved GPCR throughout vertebrate evolution and is expressed abundantly in brain structures exhibiting high levels of plasticity, e.g., the hippocampal dentate gyrus. Here, we show that SREB2 is involved in determining brain size, modulating diverse behaviors, and potentially in vulnerability to schizophrenia. Mild overexpression of SREB2 caused significant brain weight reduction and ventricular enlargement in transgenic (Tg) mice as well as behavioral abnormalities mirroring psychiatric disorders, e.g., decreased social interaction, abnormal sensorimotor gating, and impaired memory. SREB2 KO mice showed a reciprocal phenotype, a significant increase in brain weight accompanying a trend toward enhanced memory without apparent other behavioral abnormalities. In both Tg and KO mice, no gross malformation of brain structures was observed. Because of phenotypic overlap between SREB2 Tg mice and schizophrenia, we sought a possible link between the two. Minor alleles of two SREB2 SNPs, located in intron 2 and in the 3′ UTR, were overtransmitted to schizophrenia patients in a family-based sample and showed an allele load association with reduced hippocampal gray matter volume in patients. Our data implicate SREB2 as a potential risk factor for psychiatric disorders and its pathway as a target for psychiatric therapy.

gene manipulation | memory | SNPs

The SREB (superconserved receptor expressed in brain) family of SREB1 (GPR27), SREB2 (GPR85), and SREB3 (GPR173) is a unique subfamily of G protein-coupled receptor (GPCR) selectively expressed in neurons (1–5). Intriguing features of the SREB family include its high degree of sequence conservation throughout vertebrate evolution and its abundant expression in brain structures showing high levels of plasticity, for example the hippocampal dentate gyrus. Among these three members, SREB2 is the most conserved—the primary amino acid sequence is 100% identical among humans, rats, and mice. SREB1 and SREB3 are also highly conserved in mammals. Despite the extraordinary conservation rate in vertebrates, SREB orthologues are not encoded in the genome sequence of *Caenorhabditis elegans* or *Drosophila melanogaster* (3).

The history of drug discovery has proven that GPCRs are excellent therapeutic targets (6, 7). Although efforts have been made to identify endogenous ligand(s) for SREB, they have been unsuccessful (3). Recent progress in understanding of GPCR physiology has, however, enabled screening of drug candidates for promising GPCRs without knowledge of their endogenous ligands, e.g., screening compounds by using constitutively active mutants (8) or ligand-induced conformational change (9). Thus, if their physiological function is clarified, and their link to the pathophysiology of diseases is demonstrated, then newly discovered GPCRs, even orphan GPCRs like SREB2, become promising drug targets. The distinct features of SREB2, namely its unique evolutionarily conservation, its expression in virtually all neurons, and its potential role in neural plasticity (4), have led us to a working hypothesis that the SREB2 ligand-receptor system is critical in regulating neural integrity and therefore either directly or indirectly involved in the pathophysiology of CNS diseases. The neural environment could be affected by subtle changes of SREB2 activity or expression levels, because mammalian evolution has not allowed even 1 aa substitution in SREB2 protein (3).

In the present study, we generated and analyzed SREB2-overexpressing transgenic (Tg) mice and SREB2 KO mice to gain insight about SREB2 function and its possible implications in CNS disorders. We found SREB2 to be determinant of brain size and a potential factor in the etiology and pathophysiology of psychiatric disorders, especially schizophrenia.

Results and Discussion

Influence of SREB2 on Brain Size in Mice. SREB2 overexpression was achieved under the control of a calcium/calmodulin kinase II (CaMKII) promoter, by which the targeted gene is selectively expressed postnatally in forebrain neurons, i.e., cerebral cortex and hippocampus (10). In the SREB2 Tg mice, levels of SREB2 mRNA were modestly (≈2-fold over WT) increased in forebrain but the

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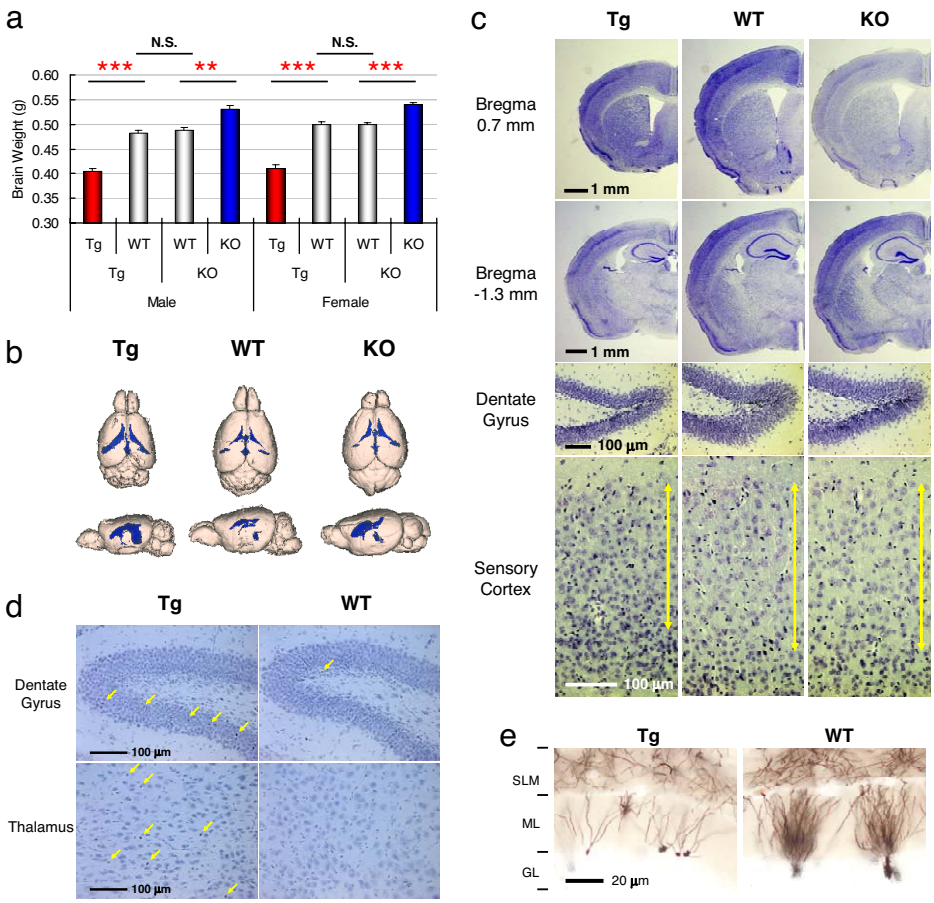

Fig. 1. Brain size was reduced in SREB2 Tg mice and increased in SREB2 KO mice. (*a*) Brain weight of SREB2 Tg and KO mice and their WT littermates (5 months old, $n = 10$). Body weight was as follows: Tg line, Male WT, 31.1 ± 2.8 g vs. Tg, 29.6 ± 1.3 g, $P = 0.13$; Female WT, 25.7 ± 0.9 g vs. Tg, 24.7 ± 0.8 g, $P < 0.05$; KO line, Male WT, 35.7 ± 2.5 g vs. KO, 31.3 ± 2.6 g, $P < 0.01$; Female WT, 28.2 ± 2.6 g vs. KO, 25.4 ± 4.4 g, $P = 0.10$. **, $P < 0.01$; ***, $P < 0.001$. (*b*) Representative magnetic resonance images of brains from SREB2 Tg mice, WT littermates (from a Tg line), and SREB2 KO mice. Parenchyma and ventricular volume was as follows: Tg line (5 months old, $n = 6$ male and 6 female), whole brain WT, 503 ± 43 mm^3 vs. Tg, 403 ± 48 mm^3, $P < 0.001$; cerebellum WT, 55.4 ± 4.3 mm^3 vs. Tg, 53.3 ± 4.6 mm^3, $P = 0.27$; ventricle WT, 2.11 ± 0.87 mm^3 vs. Tg, 3.87 ± 2.2 mm^3, $P < 0.05$; KO line (5 months old, $n = 3$ male and 3 female), whole brain WT, 484 ± 45 mm^3 vs. KO, 555 ± 49 mm^3, $P < 0.05$; cerebellum WT, 57.5 ± 6.2 mm^3 vs. KO, 71.7 ± 3.3 mm^3, $P < 0.001$; ventricle WT, 2.48 ± 0.78 mm^3 vs. Tg 3.84 ± 1.99 mm^3, $P = 0.15$. (*c*) Representative brain images of coronal sections from SREB2 Tg mice, WT littermates and SREB2 KO mice (5 months old, male). WT littermates derived from a Tg line were used. Sections were Nissl stained. Note that no gross malformation in SREB2 Tg and KO mice brains are seen. Yellow arrows show layers II/III in the sensory cortex. Neuronal soma size in the cortical area of SREB2 Tg mice was significantly smaller compared with that of WT littermates (averaged vertical length of 15 neurons from three subjects, WT, 13.7 ± 1.4 μm vs. Tg, 11.2 ± 1.1 μm, $P < 0.01$). Note that neuronal density in cortical layers II/III was higher in SREB2 Tg mice than in WT littermates. (*d*) Representative TUNEL images of forebrains from SREB2 Tg mice and WT littermates at postnatal day 10. SREB2 Tg mice showed a significant brain weight reduction during the critical developmental period (postnatal day 10, WT, 0.37 ± 0.01 g vs. Tg, 0.29 ± 0.01 g, $P < 0.001$, $n = 3$). Note that apoptotic neurons stained brown were excess in SREB2 Tg mice. (*e*) Representative Golgi staining images of hippocampal granular neurons from SREB2 Tg and WT littermates (10 months old, male). Note that fewer dendritic arborizations are seen in SREB2 Tg mice neurons. GL, granular layer of dentate gyrus; ML, molecular layer of dentate gyrus; SLM, stratum lacunosum-moleculare.

overexpression was minimal in cerebellum compared with WT littermates [supporting information (SI) Fig. S1]. SREB2 Tg mice had reduced brain weight (≈20%), whereas their body weight was not changed (male) or only slightly reduced (female) (Fig. 1*a*). Overexpression of SREB2 protein in brains of SREB2 Tg mice was also confirmed by means of mass spectrometry (M. Yuri, M.H., S. Morita, H.Y., Masanori Naitou, S.-i.M., M.M., Keitaro Mori, and Toshio Teramura, unpublished work). Magnetic resonance imaging (MRI) analysis of these mice revealed reduced whole-brain parenchyma volume but no change in cerebellum, whereas ventricular volume was significantly increased (Fig. 1*b*). Contrary to our expectation, SREB2 KO mice were viable and appeared healthy. SREB2 KO mice showed a subtle reduction (male) or a trend of reduction (female) in body weight compared with their WT littermates. Despite the slight reduction of body weight, the brain weight was significantly increased in SREB2 KO mice (≈10%, Fig. 1*a*). MRI analysis revealed increased parenchyma volume of whole brain and cerebellum in SREB2 KO mice. The effect in the cerebellum was expected because of relatively high levels of SREB2 mRNA in this brain region (Fig. S1) (1–3). Their ventricular volume showed a trend toward increase but differences were not statistically significant (Fig. 1*b*). The brain was the only organ that showed a weight gain in SREB2 KO mice (see Table S1).

Macroscopic analysis using Nissl staining revealed no gross structural abnormalities in laminar formation of cerebral cortices or position of major brain nuclei in adult SREB2 Tg mice brains. Microscopically, however, we noticed that neurons in cerebral cortices of SREB2 Tg mice were smaller and accordingly more packed than those of WT littermates (Fig. 1*c*). The brain weight of SREB2 Tg mice and their WT littermates was not different at birth, whereas 10 days after birth, a significant reduction was already evident. The first 2 weeks after birth in mice is the brain-growth spurt period when neurons are making connections and forming networks through extensive synaptogenesis. If neurons fail to be integrated in the network, they die via programmed cell death (11). During this critical time window of development, we observed excess neuronal apoptosis in forebrains of SREB2 Tg mice compared with their WT littermates (Fig. 1*d*), presumably related to a failure in the integration process because neurons had demonstrably fewer dendritic arborizations in SREB2 Tg mice brains (Fig. 1*e*). The present data suggest that downsizing of neurons and developmental neuronal loss contribute to the brain weight reduction of SREB2 Tg mice. We did not observe recognizable differences in brain structure of adult SREB2 KO mice (Fig. 1*c*) or in the occurrence of apoptosis in neonatal SREB2 KO forebrains (data not shown) from their WT littermates by standard macroscopic and microscopic analyses.

Influence of SREB2 on Behaviors in Mice. Although SREB2 Tg mice showed no apparent deficits in general motor coordination or in explorative locomotor activity (Dataset S1), they manifest behavioral abnormalities that have been used as animal models of

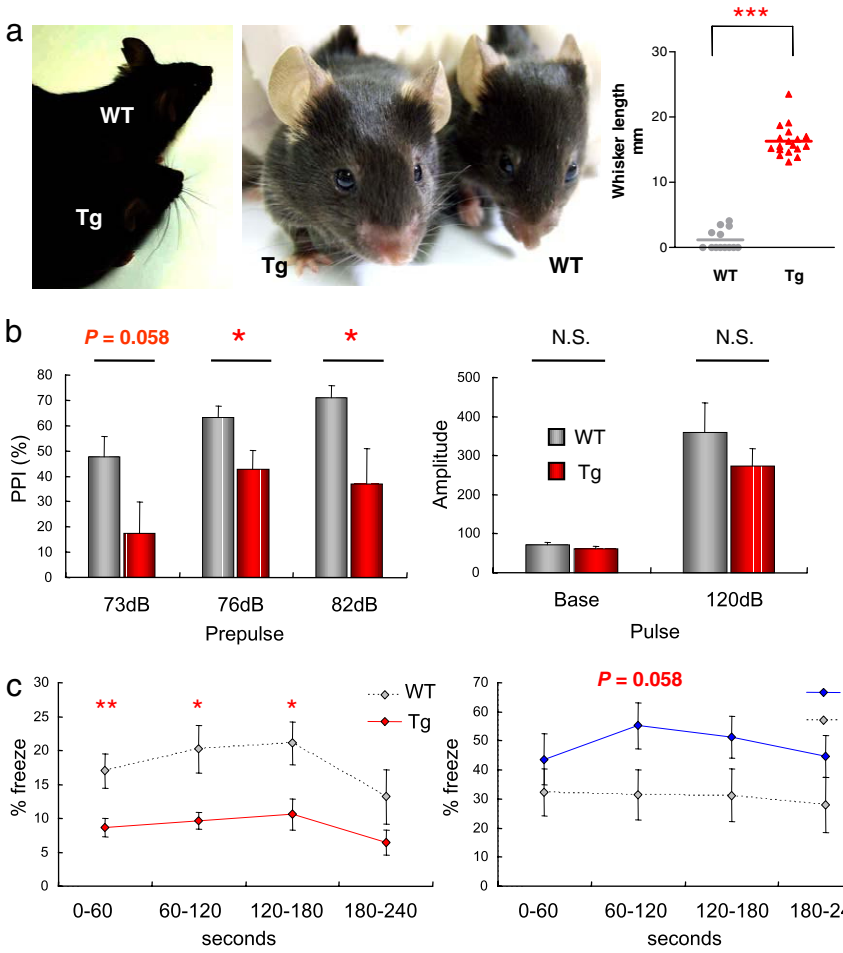

Fig. 2. SREB2 Tg mice showed behavioral abnormalities associated with psychiatric diseases. (*a*) Longer facial whiskers of SREB2 Tg mice were presumably caused by decreased social interaction compared with their WT littermates (4 months old, male). Whisker length of SREB2 Tg mice and WT littermates was not different at 2 months before the separation by their genotype (WT, 19 ± 4 mm vs. Tg, 20 ± 4 mm, $P = 0.36$). ***, $P < 0.001$. (*b*) Disruption of PPI of acoustic startle response in SREB2 Tg mice. PPI assay using a combination of startle (120 dB) and three prepulse levels (73, 76, and 82 dB). PPI is expressed as the mean percentage reduction in startle amplitude at all three prepulses. Startle amplitude was not different between SREB2 Tg mice and WT littermates. *, $P < 0.05$. (*c*) Contextual memory was impaired in SREB2 Tg mice and enhanced in SREB2 KO mice, demonstrated by fear-conditioning task. *, $P < 0.05$, **, $P < 0.01$.

neuropsychiatric disorders. We noticed that SREB2 Tg mice had longer facial whiskers compared with those of their WT littermates, who trimmed their whiskers very shortly after being housed in groups of five same-sex/genotype littermates per cage. C57BL mice, the background strain of SREB2 Tg mice, are known to trim each others whiskers as a social behavior under certain circumstances (12). Because there was no difference in whisker length between the two groups before the separation by their genotype (2 months old), the longer whiskers in SREB2 Tg mice were thought to be due to their decreased social interactions, i.e., less trimming (Fig. 2*a*). The same whisker phenotype was observed in *Dvl1* KO mice and was proposed as a model of psychiatric disorders showing social withdrawal (13). Indeed, social interaction (contact incidences in 10 min) was decreased in SREB2 Tg mice (WT 63.5 ± 2.5 vs. Tg 52.6 ± 3.3, $P < 0.05$). Deficits in attention and cognitive information processing are considered a central feature of schizophrenia, which are thought to lead to stimulus overload, cognitive fragmentation, and thought disorder (14). Prepulse inhibition (PPI) is a popular method to measure sensory information-processing deficits, and it was impaired in SREB2 Tg mice (Fig. 2*b*). In accordance with the suggested link between the SREB2 and neural plasticity (4), the fear-conditioning task revealed that SREB2 Tg mice had contextual memory deficits (Fig. 2*c*). SREB2 KO mice showed no obvious deficits in either general behaviors or PPI (Dataset S2 and Fig. S2). However, a reciprocal phenotypic tendency between the SREB2 Tg and KO mice was observed in the fear-conditioning task. Although SREB2 Tg mice failed to remember the contextual fear-conditioning stimulus, SREB2 KO mice showed a trend toward better performance in remembering the conditioning stimulus compared with their WT littermates (Fig. 2*c*).

Influence of SREB2 on Vulnerability to Schizophrenia. Several phenotypes observed in SREB2 Tg mice overlap with those seen in patients with schizophrenia, e.g., ventricular enlargement, smaller-sized cortical neurons, contextual memory deficits, social withdrawal, and abnormal sensorimotor gating (15). SREB2 is located on chromosome 7q31. Although this locus is not included among the primary susceptibility loci identified by metaanalyses of linkage studies, some reports have linked it to schizophrenia (16, 17). In addition, a patent filing reported an association between SREB2 and schizophrenia using the National Institute of Mental Health Genetics Initiative (NIMHGI) cohort (18). Thus, we performed a family-based association test (FBAT) using the Clinical Brain Disorders Branch (CBDB) Sibling Study cohort (19, 20), looking for distorted transmissions of SNP alleles from parents to affected offspring as a test for association with schizophrenia. The SNPs tested, and their linkage-disequilibrium plot are shown in Fig. S3. Overtransmission of the minor C allele for SNP M09 (second intron, $P = 0.0030$) and overtransmission of the minor G allele for SNP M01 (3′ end of third exon, $P = 0.017$) to affected individuals is evidence of association with schizophrenia (Table 1). M01 was also significantly positively associated in the case-control analysis with G/G homozygotes having a 3.17-fold-increased risk of schizophrenia versus A/A homozygotes (95% confidential interval 1.15–8.74, $P = 0.026$), consistent with the FBAT results. M09 was marginally associated with case status ($P = 0.066$). These two clinically positive SNPs also have been found to be associated with variation of amygdala and hippocampus physiological activity measured with functional (f)MRI in healthy subjects (S. Marenco, Eugenia Radulescu, J.H.C., M.M., M.F.E., R.E.S., and D.R.W. unpublished data), consistent with presumed pathophysiological

Table 1. Association of SREB2 SNPs with risk for schizophrenia

SNP	dbSNP	Major/minor	MAF	Location	Chr. 7 position	Family-based analysis	Case-control analysis			NIMHGI Wood *et al.*
							P	OR	CI	*P*
M01	rs56080411	A/G	0.174	Exon 3 3′ UTR	112314666	0.017	0.026	3.17	1.15–8.74	n.d.
M02	rs2293369	G/T	0.425	Exon 3 3′ UTR	112314775	>0.1	>0.1			n.d.
M03	rs1581688	A/G	0.377	Exon 3 3′ UTR	112317044	>0.1	>0.1			0.021
M04	rs56320353	G/A	0.022	Exon 3 3′ UTR	112317399	>0.1	>0.1			n.r.
M05	rs870266	T/A	0.059	Exon 3 3′ UTR	112317472	>0.1	>0.1			n.r.
M06	rs2256044	C/T	0.13	Exon 3 Tyr120Tyr	112318368	>0.1	>0.1			n.r.
M07	rs1608890	C/T	0.132	Exon 3 Gly84Gly	112318476	>0.1	> 0.1			n.r.
M08	rs1575012	C/A	0.27	Exon 3 5′ UTR	112318746	>0.1	> 0.1			n.r.
M09	rs56039557	G/C	0.095	Intron 2	112319584	0.0030	0.066	3.70	0.92–14.95	0.0069
M10	rs1599793	T/C	0.205	Intron 1	112321135	>0.1	>0.1			n.d.
M11	rs1599792	C/T	0.177	Intron 1	112321274	>0.1	>0.1			n.d.
M12	rs2140913	T/G	0.12	5′ upstream	112322004	>0.1	>0.1			n.d.
M13	rs1575013	C/T	0.242	5′ upstream	112322861	>0.1	0.087	2.08	0.90–4.83	n.d.
M14	rs12705846	T/C	0.388	5′ upstream	112322958	>0.1	>0.1			n.d.
M15	rs1807909	G/C	0.183	5′ upstream	112327822	>0.1	>0.1			n.d.
M16	rs7778565	C/T	0.061	5′ upstream	112328244	0.083	>0.1			n.d.
M17	rs2264670	A/T	0.053	5′ upstream	112330705	>0.1	0.089	1.64	0.93–2.89	n.d.
M18	rs10228566	C/T	0.183	5′ upstream	112331507	>0.1	>0.1			n.d.

In the study by Wood *et al.* (18), SNP S1 is our SNP M09, and SNP S7 is our SNP M03. Major/minor alleles are those located on the coding strand of the SREB2 gene [minus strand of University of California, Santa Cruz (UCSC) Genome Browser]. Chr. 7 position is in base pairs, from the UCSC Genome Browser, May 2004 Assembly. The family-based analysis *P* value is from the program FBAT. The case-control analysis *P* value is from logistic regression. CI = 95% confidence interval. The NIMHGI *P* value is from the program TRANSMIT, from the study by Wood *et al.* (18). MAF, minor allele frequency in the present cohort. OR, odds ratio of people homozygous for the minor allele being diagnosed with schizophrenia. n.d., not determined; n.r., not reported.

processes present in schizophrenia. Positive association of M09 was reported in the earlier NIMHGI family cohort analysis (18) but overtransmitted allele to the patients in this sample were different, suggesting that M09 alleles are tagging different pathogenic mutations in the different populations. Indeed, this possibility may be expected, given that virtually half the NIMHGI cohort was African American, and it also included several other ethnicity groups (18), whereas the CBDB cohort was all Caucasian (19, 20).

Compelling reciprocal phenotypes observed in SREB2 Tg and KO mice led us to investigate possible effects of the clinically positive SNPs on brain volume in patients with schizophrenia and in normal control subjects using the method of optimized MRI voxel-based morphometry (VBM). There was no effect of genotype on total brain gray-matter volume in either group. However, because SREB2 expression in mice and humans is most abundant in hippocampus throughout the entire life span (1, 2, 4), we tested for differences in hippocampal volume in humans based on SREB2 risk-associated genotypes. In patients with schizophrenia, there was a significant effect of genotype for M01, with the risk G allele having an allele load effect on reduced gray matter volume in this region ($n = 136$, $P = 0.009$, false-discovery rate corrected, Fig. 3*a*). For M09, there was a similarly significant effect, with the risk C allele load showing reduced gray-matter volume ($n = 140$, $P = 0.003$; Fig. 3*b*). Clinically negative SNPs (M11 and M12) analyzed in the same fashion showed no significant effects on hippocampal volume, providing negative controls. No significant effect of genotype at either SNP was found on hippocampal volume in normal controls, suggesting that there may be interactions with other risk factors for schizophrenia and/or medications necessary for these SREB2 risk alleles to manifest effects on hippocampal volume.

Given the evidence that SREB2 is involved in determining brain size and aspects of schizophrenia-associated pathophysiology, one might speculate a possible link between SREB2 and the Akt/PKB pathway, which is critical for neuronal growth and development and also has been implicated as a risk factor in schizophrenia (21–25). Thus, Akt activity might be predicted to be decreased in brains of SREB2 Tg mice and increased in brains of SREB2 KO mice. From our preliminary studies looking at phosphorylation levels of proteins in the Akt/PKB pathway, however, we could not find evidence

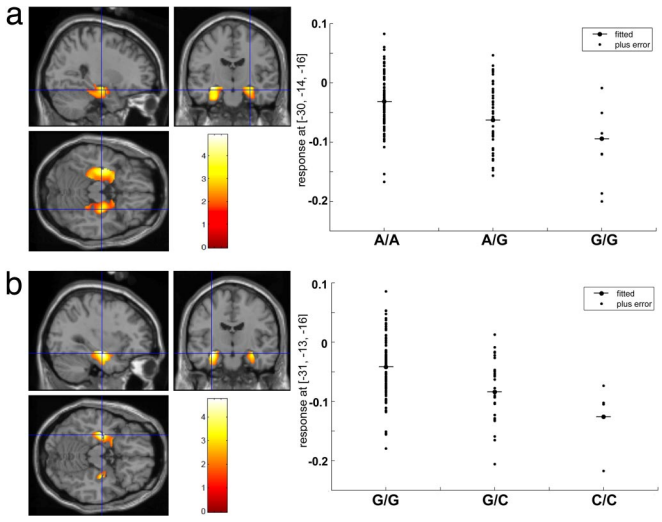

Fig. 3. Structural MRI data demonstrate hippocampal volume reduction in patients with schizophrenia carrying SREB2 risk alleles. (*a*) Effect of genotype at M01 on hippocampal volume determined with MRI VBM. G alleles are significantly associated in an allele load pattern with reduced hippocampal volume bilaterally. (*b*) Effect of genotype at M09 on hippocampal volume determined with MRI VBM. C alleles are significantly associated in an allele load pattern with reduced hippocampal volume bilaterally. Colors in the brain images represent *t* values obtained from the contrast that tests whether the slope of the regression is different from zero. Numbers on the *y* axis are residuals of the brain volume at the voxel location shown on the *y* axis after accounting for the covariates mentioned in the text.

that supports such mechanisms in adult brains (data not shown). Interestingly, levels of Akt phosphorylation at serine 473, which correlate with its activation state, were increased in brains of SREB2 Tg mice on postnatal day 10, when their brains start to decrease in weight (Fig. S4). On the other hand, SREB2 may influence brain size independent of the Akt/PKB pathway, and we might have observed a compensational response of the Akt/PKB pathway in the SREB2 Tg neonatal brains.

Although 7q31 is not considered a consistent schizophrenia linkage locus, it has been more consistently implicated in linkage studies of other psychiatric disorders, e.g., autism (26), speech and language disorders (27), and Tourette's syndrome (28). Behavioral deficits and morphological brain abnormalities observed in SREB2 Tg mice are also observed in these psychiatric disorders. Importantly, mRNA levels of SREB2 are dynamically regulated *in vivo*, e.g., they strongly increase after overstimulation of the glutamate system, as in kainate-induced seizures (2). Thus, dynamic changes of SREB2 gene expression could be tightly regulated through many checkpoints. Indeed, not only the coding region but also noncoding regions of the SREB2 gene are highly conserved in mammals (http://genome.ucsc.edu), which suggests that funtional *cis* elements involved in transcriptional and/or posttranscriptional control are encoded in these regions and that SNPs in noncoding regions might affect expressional regulation (SNPs and transcription factor-binding sites are summarized in Dataset S3). Our present data warrant further studies investigating the possible involvement of SREB2 in brain disorders. Toward this end, specific agonists and antagonists for SREB2 should be powerful tools and may eventually lead directly to the development of therapeutics for neuropsychiatric disorders.

Materials and Methods

Generation of SREB2 Tg and KO Mice. SREB2 Tg mice were generated by overexpressing the mouse SREB2 gene (the whole fourth exon encoding its entire intronless ORF, ≈4.5 kb from the start codon) driven by the mouse CaMKII promoter (≈8 kb) as described (10). The expression construct was injected into the pronuclei of fertilized eggs from a BDF1 mouse and the generated Tg mice were maintained on a C57BL/6 background (Japan SLC). A Tg line overexpressing SREB2 (≈2-fold over WT) showed brain weight reduction. Another independent Tg line (≈4-fold over WT) showed the same brain weight reduction (≈25%), confirming that the brain effect was due to SREB2 overexpression. In this study, we used heterozygotes of the F_3 or F_4 generations from the Tg line (≈2-fold over WT) unless otherwise specified, and comparisons were made with littermate WT mice in all experiments. SREB2 KO mice were generated as described (29). To construct a SREB2 targeting vector, the long arm of the 7-kb region, which includes exon 1 to exon 3, and the short arm of the 2-kb region, which includes exon 4 and the 3′ UTR of the mouse SREB2 gene were ligated into the targeting vector pPNT. The targeting vector was transfected into TT2 cells by electroporation, and clones resistant to G418 and ganciclovir were selected and further screened by Southern blot hybridization. Chimeric male mice were mated with C57BL/6 females to obtain F_1 heterozygotes (Trans Genic). F_1 heterozygous mice were crossed with C57BL/6 mice to produce a large number of F_2 heterozygous mice, which were then intercrossed to produce homozygous mice for analysis. Quantitative RT-PCR detected no SREB2 mRNA in brains of SREB2 KO mice. In this study, we used homozygous mice of the F_3 generations, and, in all experiments, comparisons were made with littermate WT mice. DNA was extracted from tails of mice and genotyped by PCR with the following primer sets; SREB2 primers for both Tg and KO (5′-GCACGAGGGCCTGTAGTACC-3′ and 5′-CATCCAGACAGCGGCTGTTA-3′) and neomycin primers for KO (5′-TATGGGATCGGCCATTGAAC-3′ and 5′-CCTCAGAAGAACTCGTCAAG-3′). All experiments were performed in compliance with the regulations of the Animal Ethics Committee of Astellas Pharma, Inc.

Brain Biochemical/Morphological Analysis. Analysis of SREB2 Tg/KO mice brains was performed by using WT littermates' brains as a control. Differences between groups were examined for statistical significance by using Student's two-tailed unpaired *t* test. Results are expressed as the mean ± SD. Details are given in *SI Materials and Methods*.

Behavioral Analysis. Male SREB2 Tg mice, SREB2 KO mice, and their male WT littermates (3 months of age or older, $n = 10$) were used in all experiments. Differences between groups were examined for statistical significance by using Student's two-tailed unpaired *t* test. Results are expressed as the mean ± SEM.

Social-Interaction Test. For the social-interaction test, every SREB2 Tg/WT mouse was randomly assigned to an unfamiliar partner in each genotype group. Each pair of unfamiliar mice was placed in the test apparatus (50 × ≈50 × ≈40 (H) cm) for 10 min, and the number of contacts was counted. Behavior was recorded on a Macintosh computer and analyzed by using Image Text 1.10 (O'Hara and Co.), a software based on the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

Prepulse Inhibition (PPI) Test. We used a startle response-measuring system for small animals to determine PPI (SR-LAB; San Diego Instruments) Details are given in *SI Materials and Methods*.

Fear Conditioning Test. Each mouse was placed into an apparatus for measuring fear conditioning (Acti Metrics) based on an electrical unconditioned stimulus. The mice were then administered an electrical stimulus (0.4 mA for 1 s) every 15 s for 4 min. The next day, the mice were again placed into the experimental apparatus, and no electricity was administered. Conditioned fear was recorded as positive if the animal remained immobile for the 4-min test period.

Human Subjects and Genotyping. A family-based sample was ascertained as part of the Clinical Brain Disorders Branch/National Institute of Mental Health (NIMH) Sibling Study (20, 30, 31). We examined families with a proband with schizophrenia ($n = 358$). A nonindependent case-control study comprised 358 unrelated probands and 370 unrelated healthy controls. Subjects were all of European ancestry to minimize genetic heterogeneity and stratification artifacts. All subjects were from 18 to 60 years of age, were >70 in IQ, and gave written informed consent. Exclusion criteria were significant medical problems, history of loss of consciousness for >5 min, alcohol or drug abuse/dependence within the last 12 months, and electroconvulsive therapy within the last 6 months. All subjects were interviewed by a research psychiatrist using the Structured Interview for DSM-IV. For probands, data from psychiatric records were also evaluated during diagnostic ascertainment. The study was approved by the NIMH Institutional Review Board. Near-complete resequencing of introns and exons of SREB2 and 2 kb upstream of its transcription start site was accomplished in 48 individuals with schizophrenia. A previously uncharacterized SNP in the 3′ UTR (M01, ss73689505) was found, but we observed no mutations that directly alter the amino acid sequence (see Table 1). By using the TaqMan 5′-exonuclease allelic discrimination assay, all identified SNPs with minor allele frequencies >3% were genotyped in the clinical samples, along with HAPMAP tag SNPs and SNPs reported to be associated with schizophrenia. The 18 SNPs tested were located from ≈10 kb upstream of the first known transcribed exon to the 3′ end of the last known transcribed exon (exon 3). Deviation from Hardy–Weinberg Equilibrium (HWE) was tested by using exact tests.

Statistical Analysis of Clinical Association. Analyses of single SNPs in families were conducted by using the Family Based Association Test (FBAT) (32), a method that looks at observed transmissions of alleles or haplotypes from parents to affected offspring vs. expected transmissions under the null hypothesis. Significance was determined with a permutation test based on 1,000 permutations. Case-control analyses were conducted by using logistic regression. Most of the cases were taken from the families that were part of the FBAT analysis. *P* values were not adjusted for multiple testing in any sample. A *P* value of 0.05 was taken as the threshold for significance for this initial exploratory analysis, however, some of the SNPs tested had already shown positive association with schizophrenia in the NIMHGI sample (18) and should be considered replications.

Brain Imaging. Three-dimensional structural MRIs were obtained in a large collection of patients with schizophrenia and controls on a 1.5-tesla GE scanner using a T1-weighted SPGR sequence (repetition time, 24 msec; echo time, 5 msec; excitations, 1; flip angle, 45°; matrix size, 256 × 256; field of view, 24 × 24 cm), with 124 sagittal slices at a thickness of 1.5 mm and an in-plane resolution of 0.94 × 0.94 mm. To analyze effects on whole-brain gray matter and hippocampal volume, we used optimized VBM performed in SPM2, including the nonuniformity correction option (33). Images were modulated by multiplication with the Jacobian determinant of the spatial normalization function, and an integration of modulated gray matter voxel values resulted in estimates for total gray matter volume. Modulated images were smoothed by using an 8-mm FWHM isotropic Gaussian kernel. The normalized, modulated, and smoothed gray matter images were analyzed by using the General Linear Model as implemented in SPM2. For the analysis of differences between genotypes, we used separate regression analyses in controls and patients with schizophrenia, with genotype group

(homozygotes for the common allele and heterozygotes and homozygotes for the uncommon allele) as a factor, and age, second-order polynomial age expansions, gender, and IQ as covariates of no interest. Total gray matter volume differences across genotypes were assessed by means of an ANOVA. Total gray matter volume was then used as a covariate for the analysis of regional hippocampal volume. Automatic regions of interest (ROIs) from the Wake Forest University (WFU) Pickatlas (www.fmri.wfubmc.edu) (34) were used to extract hippocampal volume across genotypes, therefore reducing the number of multiple comparisons and allowing for stringent small volume correction. The threshold for significance was $P = 0.05$, false-discovery rate corrected, with a cluster size >3 contiguous voxels. We have shown that this approach to statistical correction is a conservative strategy for eliminating false positives in imaging analyses of genotype effects on brain structure (35).

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