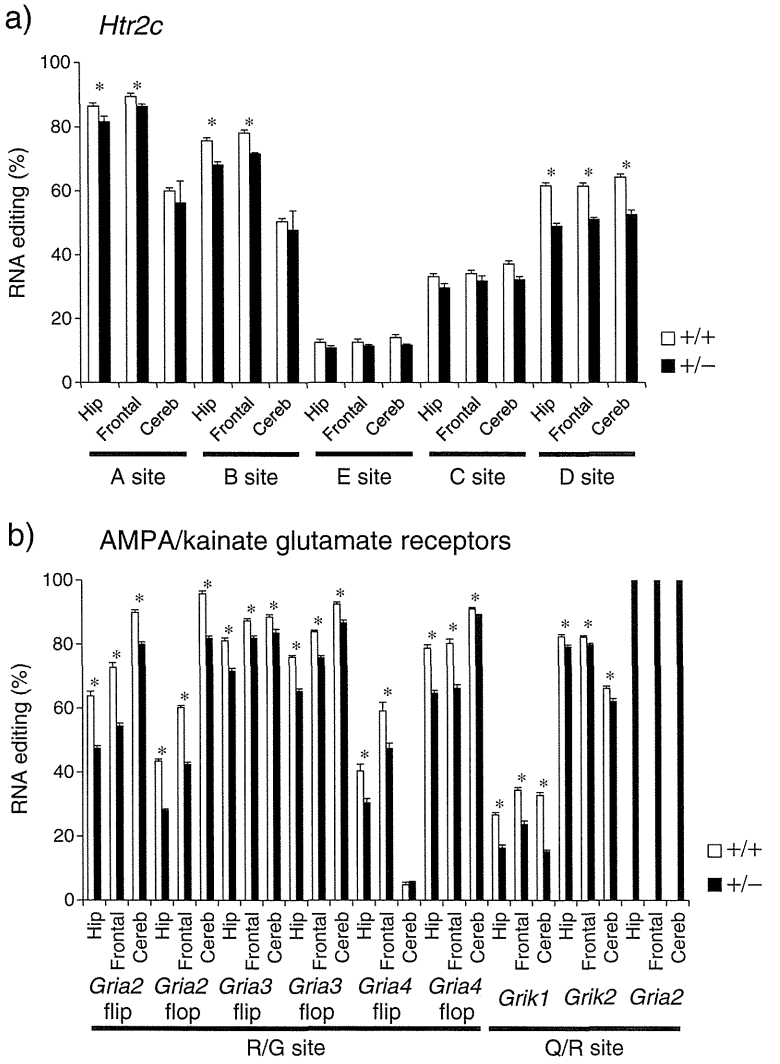

Figure 1 RNA editing of *Htr2c* and AMPA/kainate glutamate receptors in *Adar2*$^{+/-}$ mice. a) *Htr2c*. **b)** AMPA/kainate glutamate receptors. $P < 0.05$ by Student *t*-test. Hip denotes hippocampus, Frontal denotes frontal cortex, and Cereb denotes cerebellum. White bars indicate the WT mice, black bars indicate the *Adar2*$^{+/-}$ mice. Data represent mean and error bars indicated by standard errors (n = 10 for each genotype).

Gria3, and *Gria4*). The R/G site showed a 10–36% decrease except for *Gria4* in the cerebellum, where minimal or no alteration in editing efficiency was observed.

RNA editing in human postmortem brains

We previously reported RNA editing efficiency of *HTR2C* in the Consortium samples, which showed an increase at the D site in depression and an increase at the A site in suicide completers [12]. In this study, we measured the editing efficiency of AMPA/kainate receptors in the same sample set. As expected, the Q/R sites were almost fully edited in all groups. There was no significant alteration in RNA editing of R/G sites, but there was a tendency toward decreased editing efficiency in mood disorders (Table 3). The editing efficiency at the R/G site was significantly correlated with the *ADAR2* expression level for all transcripts investigated ($r = 0.30$–0.64, $P < 0.05$ for *GAPDH*-normalized and $r = 0.25$–0.64, $P < 0.05$ for *CFL1*-normalized data) (Table 3); however, the Q/R site of *GRIA2* was not ($P > 0.05$). As shown in Figure 2, some of the patients showed a prominent decrease in both editing efficiency of R/G sites and *ADAR2* expression levels.

Behavioral analysis of *Adar2*$^{+/-}$ mice

The present findings suggest that *ADAR2* is downregulated in schizophrenia and bipolar disorder, which correlates with decreased R/G site editing of AMPA receptors. Thus, decreased *ADAR2* levels and the resultant alteration of editing efficiency at the R/G sites of AMPA receptors might have some pathophysiological significance in mood disorders and schizophrenia. To test this,

Table 3 RNA editing of AMPA receptors in the postmortem brains of patients with mental disorders

	R/G sites						Q/R sites
	***GRIA2* flip**	***GRIA2* flop**	***GRIA3* flip**	***GRIA3* flop**	***GRIA4* flip**	***GRIA4* flop**	***GRIA2***
Bipolar disorder	64.4 ± 16.4	54.4 ± 12.5	66.3 ± 9.5	79.4 ± 10.6	56.0 ± 17.1	64.8 ± 15.8	96.6 ± 3.4
Depression	62.5 ± 13.8	54.9 ± 5.3	61.9 ± 9.6	82.6 ± 7.7	55.0 ± 15.8	69.9 ± 10.7	97.6 ± 3.0
Schizophrenia	66.0 ± 10.8	53.7 ± 8.0	66.0 ± 5.9	83.5 ± 9.4	60.5 ± 9.7	71.5 ± 6.1	99.0 ± 1.9
Control	70.1 ± 4.8	58.1 ± 4.4	65.0 ± 12.1	85.5 ± 3.5	63.2 ± 9.5	71.2 ± 7.5	98.8 ± 2.3
*r** (*GAPDH*)	0.508	0.476	0.300	0.644	0.527	0.565	ns
*r** (*CFL1*)	0.477	0.430	0.251	0.642	0.509	0.600	ns

**r* = Correlation coefficient between editing efficiency of AMPA receptors and *ADAR2* expression level.

we applied a battery of conventional behavioral tests to $Adar2^{+/-}$ mice. The results are summarized in Figure 3. In open-field test, RMANOVA with the intrasubject factor of time (1–20 min, df = 19) and the intersubject factor of genotype showed a trend-level effect of genotype (F = 4.0, P = 0.054) for locomotor activity. The $Adar2^{+/-}$ mice tended to be more hyperactive than the wild-type (WT) mice (Figure 3a, t = 2.01, P = 0.05). No significant difference was found in the rearing scores (Figure 3b). There was no alteration in prepulse inhibition (Figure 3c), which does not support the hypothesis that $Adar2^{+/-}$ mice show schizophrenia-like sensorimotor gating abnormality. Among the factors affecting the results of the open-field test, a possible effect of anxiety was not supported because there was no significant alteration by the elevated plus maze (Figure 3d, 3e). The Morris water maze test did not show any difference, indicating that there was no marked impairment in spatial memory (Figure 3f, 3g). No significant alteration in passive avoidance test (Figure 3h) is in accordance with lack of significant alteration in elevated plus maze test. In the active avoidance test, there was no significant time × genotype interaction in the avoidance latency or number of avoidances by repeated measures (RM) ANOVA (RMANOVA) (Figure 3i, 3j). In the forced swimming test (Figure 3k, 3l), the ratio of the immobility time on the second day to that of the first day, which is an indicator for behavioral despair, tended to be smaller in the $Adar2^{+/-}$ mice (t = 1.9, P = 0.06). This suggests that $Adar2^{+/-}$ mice showed a tendency of resistance to behavioral despair.

Pharmacological experiments

To further elucidate the mechanism of increased locomotor activity in $Adar2^{+/-}$ mice, we examined the effect of amphetamine administration in the mice. Amphetamine is known to evoke a delayed overflow of glutamate in the brain in addition to having an impact on the dopaminergic system. It causes acute hyperactivity and behavioral sensitization [22,23]. Less editing in the GluR2 receptors would result in high Ca^{2+} permeability in AMPA receptors, which may enhance glutamate

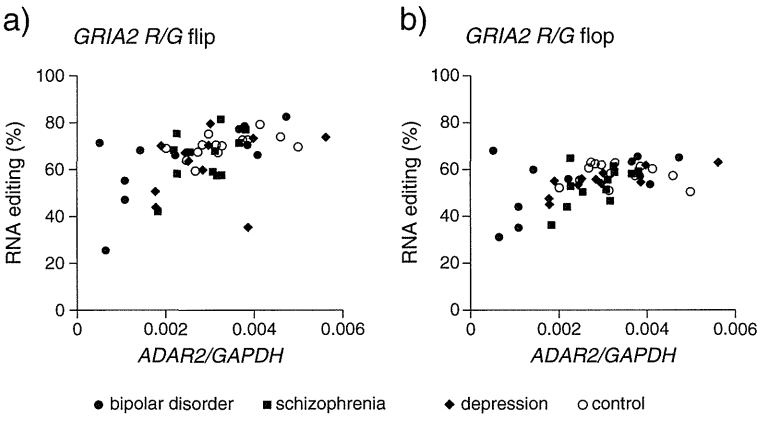

Figure 2 RNA editing of *GRIA2* R/G site and *ADAR2* expression level in human postmortem brains. Relationship between expression level of *ADAR2* and editing efficacy at R/G sites of *GRIA2*. **a)** *GRIA2*, flip isoform. **b)** *GRIA2*, flop isoform. The vertical axis means the RNA editing efficiency at the R/G site of *GRIA2*. The horizontal axis shows the mRNA expression of *ADAR2* shown by the *ADAR2/GAPDH* ratio. Each dot represents the one person's editing efficacy at the R/G site of *GRIA2* versus their expression level of *ADAR2*. Closed circles represent bipolar disorder (n = 11), closed squares represent schizophrenia (n = 13), closed diamonds represent depression (n = 11), and open circles represent controls (n = 14). Correlation coefficients were r = 0.53 (P < 0.001) for the flip isoform and r = 0.42 (P < 0.005) for the flop isoform. The information of each subject was shown in Neuropathology Consortium samples of Table 1.

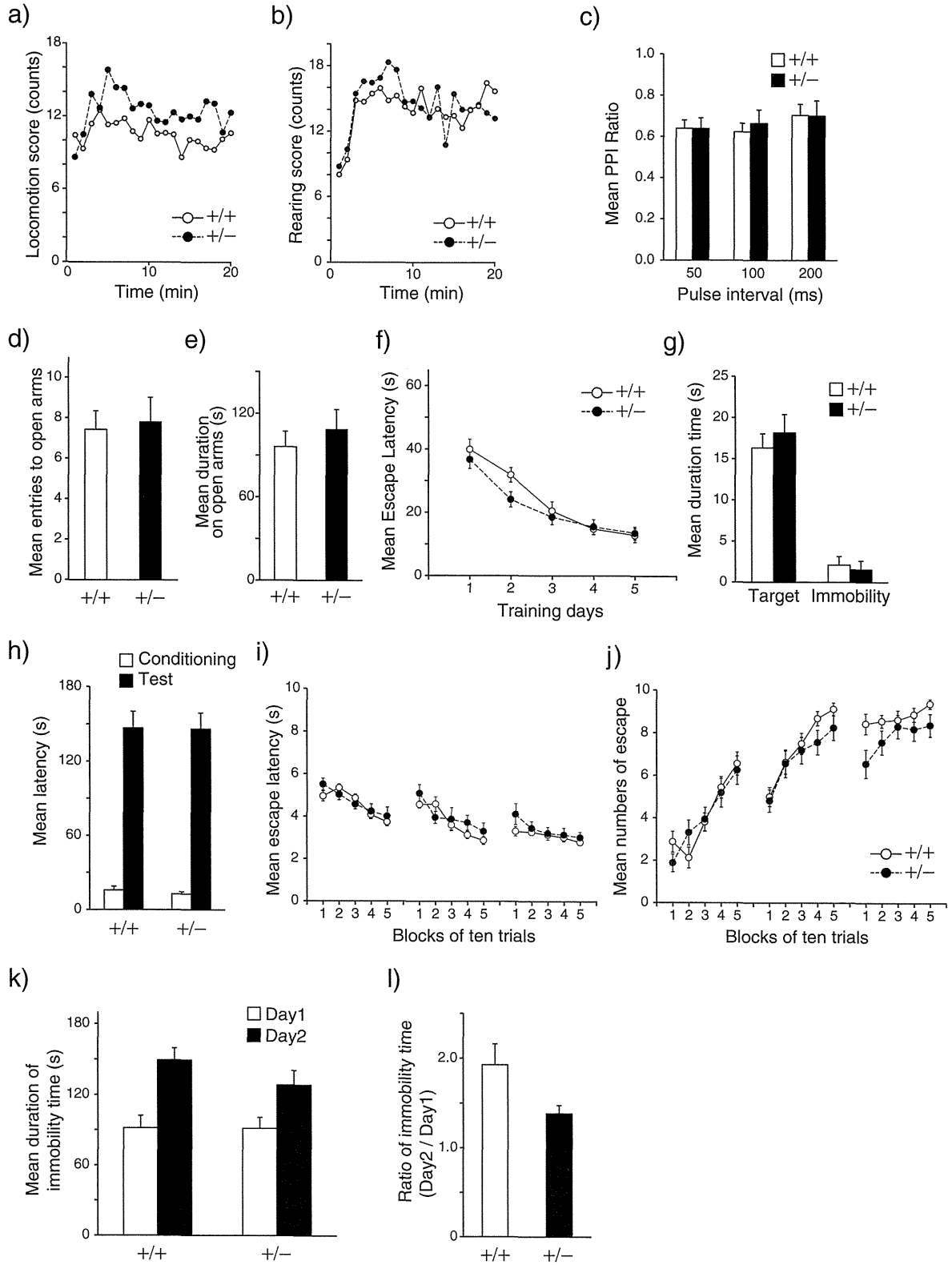

Figure 3 (See legend on next page.)

(See figure on previous page.)
Figure 3 Behavioral battery in *Adar2*$^{+/-}$ mice. a,b) Open-field test. The vertical axis is the counts measured by the interruption of infrared beams. **a)** Locomotor activity. **b)** Rearing. Closed circles indicate the *Adar2*$^{+/-}$ mice (+/–). Open circles indicate the WT mice (+/+). No significant genotype × time interaction was found for locomotor activity. Locomotor count tended to be higher in the *Adar2*$^{+/-}$ mice than that in the WT mice (t = 2.0, P = 0.05). RMANOVA with the intrasubject factor of time (1–20 min, df = 19) and the intersubject factor of genotype showed no significant effect of genotype or genotype × time interaction in rearing count. **c)** PPI test. There was no significant difference between genotypes. **d,e)** Elevated plus maze test. **d)** Number of entries to open arms. **e)** Time spent on open arms. There was no significant difference in the number of entries and time spent on open arms between genotypes. **f,g)** Morris water maze test. **f)** Escape latency. No significant effect of genotype was found for the escape latency by RMANOVA. **g)** Probe test. There was no significant difference between genotypes. **h)** Passive avoidance test. There was no significant effect of genotypes by two-way ANOVA. **i,j)** Active avoidance test. There was no significant interaction between time (df = 14) and genotype (df = 1) in the avoidance latency **(i)** or number of avoidances **(j)** by RMANOVA (P = 0.05). **k,l)** Forced swimming test. **k)** Duration of immobility. **l)** The ratio of immobility time on the second day to the first day. Data represent mean and error bars indicated by standard errors (n = 16 for each genotype).

transmission in *Adar2*$^{+/-}$ mice. Previously, it was shown that the AMPA receptor antagonist NBQX by itself had no effect on locomotor activity but prevented hyperactivity after treatment with amphetamine [24,25]. In accordance with previous studies, the locomotor activity gradually declined in two groups with treatment of saline or NBQX in both the WT and the *Adar2*$^{+/-}$ mice in our experiment. There was no significant difference of activity level between saline injection and NBQX injection both in the WT mice and the *Adar2*$^{+/-}$ mice [see Additional file 1: Figure S1]. Thus, we examined this behavioral trait in relation to amphetamine treatment of *Adar2*$^{+/-}$ mice. The amphetamine treatment enhanced the activity level in both the WT and the *Adar2*$^{+/-}$ mice (Figure 4a, 4b). RMANOVA showed significant effects of genotype (df = 1, F = 17.8, P <0.005) and drug (df = 1, F = 70.9, P < 0.001), as well as a significant interaction of genotype × drug (df = 1, F = 7.1, P < 0.05). The enhancement was significantly larger in the *Adar2*$^{+/-}$ mice than in the WT mice (Figure 4c, t = 3.07, P = 0.015). Amphetamine treatment after the NBQX treatment also enhanced the locomotor activity in the *Adar2*$^{+/-}$ mice (9.03 ± 7.16) and the WT mice (7.51 ± 5.19) (Figure 4d, 4e). RMANOVA showed significant effects of drug (df = 1, F = 58.1, P < 0.001) but no significant effect of genotype (df = 1, F = 0.37, P=0.54) and no significant interaction of genotype × drug (df = 1, F = 0.29, P = 0.59). There was no significant difference in the enhancement between genotypes (Figure 4f, t = 0.146, P = 0.56).

Discussion

In this study, we performed an integrated analysis of *ADAR2* and RNA editing of AMPA/kainate glutamate receptors in human postmortem brains and model mice. Recent expression studies using postmortem brains showed no significant alteration of *ADAR2* [6,26] and significant down-regulation of *ADAR2* in major depressive disorder [15]. In our study, although the results were not consistent between two independent sample sets, we observed general down-regulation of *ADAR2* in the brains of patients with schizophrenia or mood disorders. The discrepancy in the results across studies may be partly due to the complexity of *ADAR2* transcripts [27]. In this study, we examined the gene expression level of *ADAR2* transcripts containing exons 11 and 12. Another notable feature was that some patients, but not all patients, showed drastic down-regulation of *ADAR2*, which was accompanied by decreased RNA editing at the R/G sites.

To model the down-regulation of *ADAR2* in patients with mental disorders, we analyzed *Adar2*$^{+/-}$ mice. Altered RNA editing of *Htr2c* was characterized by a relatively large decrease at the D site with smaller changes at the A and B sites. A decrease of D site editing in depression has been reported in some [11,14], but not all [9,10,12,15] studies. The altered RNA editing of AMPA/kainate receptors in *Adar2*$^{+/-}$ mice was characterized by preserved Q/R site editing of *Gria2* and decreased editing of R/G sites. This is consistent with a previous study using semiquantitative analysis of sequence chromatograms [21]. Although it is a limitation of the study that we did not measure the protein levels of Adar2, the alteration of RNA editing status might suggests that haploinsufficiency of *Adar2* would cause a decrease of Adar2 protein level and subsequently reduced Adar2 activity in *Adar2*$^{+/-}$ mice [21]. Because there was some residual Q/R site editing of *Gria2* in homozygous *Adar2* knockout mice, *Adar1* might also play some role in the editing of this site as well as RNA editing of the R/G site of AMPA receptors and *Htr2c*. However, *Adar1* was not upregulated in homozygous *Adar2* knockout mice, suggesting that compensatory upregulation of *Adar1* do not play a major role in RNA editing changes in *Adar2*$^{+/-}$ mice.

In the postmortem brains of patients with schizophrenia and mood disorders, statistically significant alteration of RNA editing of AMPA/kainate receptors was not found. The lack of prominent alteration of the Q/R site in schizophrenia is consistent with the pioneering work of Akbarian and colleagues [16]. However, there was a significant correlation between *ADAR2* expression and R/G site editing. This suggests that decreased *ADAR2*

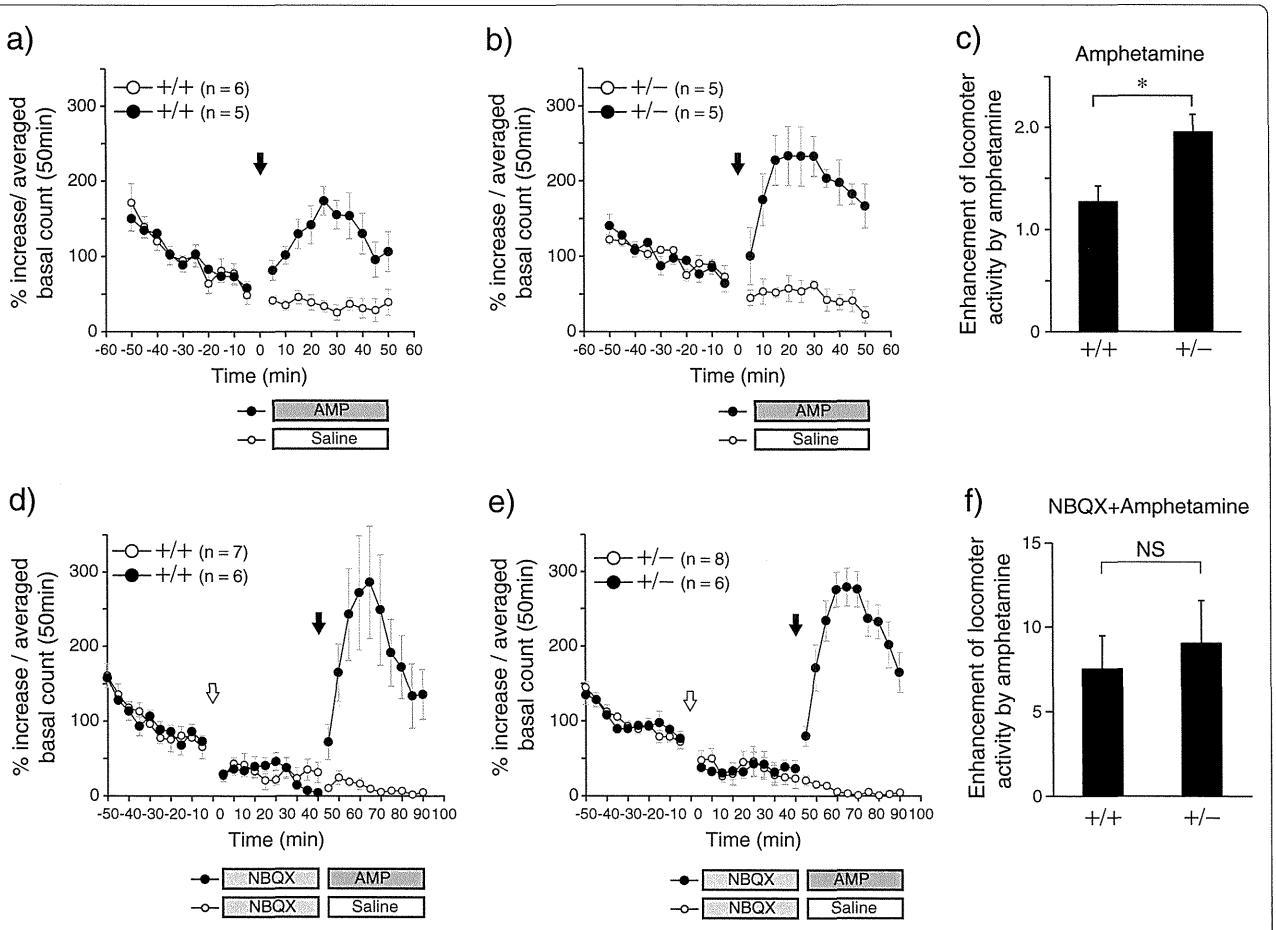

Figure 4 Effects of amphetamine with or without NBQX pre-treatment on locomotor activity in *Adar2*$^{+/-}$ mice. a,b) Effect of amphetamine on locomotor activity in the WT mice **(a)** and the *Adar2*$^{+/-}$ mice **(b)**. The horizontal axis indicates the time before and after administration of amphetamine or saline. The vertical axis indicates the activity level. Each dot represents the averaged activity level during the 5-min period. Closed circles indicate amphetamine-injected group and open circles indicate saline-injected group. Arrow indicates the time of drug administration. **c)** The vertical axis indicates the fold change of the averaged activity level during 50 min after the administration of amphetamine to the averaged activity during 50 min before the treatment. *P = 0.05 by Student t-test. **d,e)** Effect of NBQX on amphetamine-induced hyperactivity in the WT mice **(d)** and the *Adar2*$^{+/-}$ mice **(e)**. The horizontal axis indicates the time before and after the administration of amphetamine or saline. The vertical axis indicates the activity level. Each dot represents the averaged activity level during the 5-min period. Closed circles indicate the amphetamine injected group and open circles indicate the saline-injected group. Arrows indicate the time of drug administration. The open arrow indicates the administration of NBQX and the closed arrow indicates the injection of amphetamine or saline. **f)** The vertical axis indicates the fold change of the averaged activity level during 50 min after the administration of amphetamine to the averaged activity during the middle 50 min after the NBQX treatment. No significant difference was found between genotypes. Data represent mean and error bars indicated by standard errors.

expression in patients with mental disorders has some functional impact on RNA editing, similar to that observed in *Adar2*$^{+/-}$ mice. Indeed, the editing of R/G sites tended to be decreased in mood disorders as a whole (Table 3), and some patients showed markedly decreased editing efficiency (Figure 2). Thus, down-regulation of *ADAR2* and the resultant decreased editing of R/G sites might have some pathophysiological significance, at least in a subgroup of patients. The causes of downregulation of *ADAR2* in these disorders are unknown. A recent study searched for proteins regulating *ADAR2* and found three RNA binding proteins, RPS14, SFRS9 and DDX15 [28]. We previously performed gene ontology analysis of differentially expressed genes in the postmortem brains of patients with bipolar disorder and found that genes related to RNA binding and RNA splicing were significantly enriched [29]. Indeed, *SFRS9*, a splicing factor, was downregulated in the postmortem brain of patients with schizophrenia and bipolar disorder (unpublished finding). Thus, RNA machinery might be somehow dysregulated in bipolar disorder and schizophrenia.

Lyddon and colleagues examined the RNA editing in AMPA/kainate receptors in the same samples (i.e., the Neuropathology Consortium samples) [17]. Their finding that the flop isoform of *GRIA3* and *GRIA4* showed higher R/G site editing compared with the flip isoform

was replicated in this study. They also reported that the diagnosis did not affect the RNA editing efficiency, which is compatible with the present finding that there is no significant difference between diagnoses.

In this study, *Adar2*$^{+/-}$ mice showed slight tendencies of altered behavior by comprehensive behavioral battery. The *Adar2*$^{+/-}$ mice showed hyperactivity in the open-field test, but they did not show altered prepulse inhibition, a candidate endophenotype in schizophrenia.

Amphetamine-induced hyperactivity was significantly enhanced in the *Adar2*$^{+/-}$ mice. However, the difference in this enhancement was no longer significant after the pretreatment with NBQX, an inhibitor of the AMPA/kainate receptor. This suggests that the enhanced response to amphetamine seen in *Adar2*$^{+/-}$ mice might be mediated by the enhanced glutamatergic signaling caused by altered function of AMPA receptors due to the decreased RNA editing of these receptors. However, a possibility that NBQX enhanced amphetamine-induced hyperactivity in WT mice but not in *Adar2*$^{+/-}$ mice cannot be totally ruled out, because we did not set a control group to examine the effect of amphetamine after saline injection. Moreover, editing of *Htr2c* was also affected by haploinsufficiency of *Adar2*, and its contribution to the enhanced response cannot be ruled out.

In addition to the *HTR2C* and AMPA/kainate receptors, recent studies using next-generation sequencers showed numerous previously unidentified editing sites [30-34], although some of the initial findings could contain false-positive sites [35,36]. In spite of the controversy surrounding the bioinformatics analysis of RNA–DNA differences, many other A-to-I editing sites such as *Cyfip2*, *Kcna1*, *Blcap*, *Gabra3*, *Flna*, *Flnb*, and *Cadps*, have been experimentally validated as target editing sites of *Adar2* [37]. Thus, alteration of RNA editing of transcripts other than those for glutamate receptors and *HTR2C* can also contribute to the phenotypes observed in this study.

Horsch and colleagues performed behavioral analysis of homozygous *Adar2* knockout mice under the background of homozygous knock-in alleles of an edited version of *Gria2* to rescue severe phenotypes due to loss of Q/R site editing of *Gria2* [37]. These mice had dramatically decreased R/G site editing of *Gria2* (15%) and *Gria4* (10%) as well as profound alterations in RNA editing of *Htr2c* [21], and showed increased passive rotation in a rotarod test, impaired hearing ability, increased raring in open field test and impaired prepulse inhibition. Some of these findings might be attributable to decreased editing of *Htr2c*. *Adar2*$^{+/-}$ mice did not show impairment in hearing ability and prepulse inhibition or altered response to open field test, possibly because of milder impairment in RNA editing of *Htr2c* and/or R/G site of AMPA receptors. However, regarding the open-field test, they showed increased rearing during the first 5 min but did not show any alteration in locomotor activity [37]. This difference may be due to different methodologies; however, less extensive editing abnormality in heterozygous knockout might cause a different phenotype.

Another group generated transgenic mice expressing *Adar2* [38] and found that these mice showed increased immobility time and decreased activity in the open-field test. Pairing this information with our findings that *Adar2*$^{+/-}$ mice showed a tendency of increased activity in the open-field test and a tendency of resistance to immobility in the forced swimming test, we can postulate that the levels of *Adar2* and the resultant RNA editing changes might be related to the activity level or liability to the behavioral despair exhibited in the forced swimming test. In the *Adar2* transgenic mice, editing of the A, C, D, and E sites of *Htr2c* was also increased [39]. Together with the decreased editing of A, B, and D sites in *Adar2*$^{+/-}$ mice, it is possible that altered A and D site editing of *Htr2c* might also contribute to the behavioral phenotypes of *Adar2*$^{+/-}$ mice.

At this stage, we should be cautious about directly connecting the behavioral features of mice such as activity in open-field tests, forced swimming tests, and enhanced amphetamine response to mental disorders such as schizophrenia, depression, or mania. To extend this preliminary finding, other animal models and effects of psychotropic medications should also be examined. In spite of its limitations, the present study suggests that an altered expression level of *Adar2* due to haploinsufficiency affects the behavior of mice at least partly through the altered RNA editing efficiency of AMPA/kainate receptors.

Conclusion

In conclusion, *ADAR2* expression is decreased in the postmortem brains of patients with schizophrenia and bipolar disorder, and decreased *ADAR2* expression is correlated with decreased RNA editing of the R/G site in AMPA glutamate receptors. *Adar2*$^{+/-}$ mice showed decreased RNA editing of the R/G site of AMPA receptors. These mice showed slight behavioral changes such as hyperlocomotion in the open-field test, attenuated immobility response to the forced swimming test, and enhanced response to amphetamine. The difference of amphetamine response was not seen after the treatment with the AMPA/kainate receptor antagonist, NBQX. These findings collectively suggest a possible role of altered RNA editing efficiency of AMPA receptors due to down-regulation of *ADAR2* in the pathophysiology of mental disorders.

Methods

Postmortem brains

Two sets of postmortem brain samples donated by the Stanley Medical Research Institute were used for this

study (https://www.stanleygenomics.org) [40]. One set was the Array Collection, consisting of 104 RNA samples extracted from the prefrontal cortex (Brodmann area 46) (34 bipolar disorder, 35 schizophrenia and 35 controls). The other set comprised frozen brain tissue samples (Brodmann area 10) from the Neuropathology Consortium. They were derived from patients with bipolar disorder (n = 15), major depression (n = 15), and schizophrenia (n = 15) and 15 controls. Diagnoses had been made according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition [41]. Because of the RNA quality obtained, 101 and 49 samples from the Array Collection and Neuropathology Consortium, respectively, could be used for this study (Table 1). Subjects' demographic information is shown in Table 1. This study was approved by the Ethics Committee of RIKEN.

Animals

Adar2 knockout mice were developed by Higuchi and colleagues as described [21]. In brief, a targeting vector to replace exon 4 of *Adar2* with a PGK–neo gene was used for generation of a targeted embryonic stem cell line. Chimeric mice were generated by injection of this clone into C57BL/6-derived blastocysts, and homozygous *Adar2* knockout mice were bred thereafter. Genotyping of the mice was performed as described [21].

The mice were maintained in a 12-h light/12-h dark cycle. All animal experiments were approved by the local animal experiment committees of RIKEN and the Behavioral and Medical Sciences Research Consortium (BMSRC) (Akashi, Japan). Animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Real-time quantitative reverse transcription polymerase chain reaction analysis in human brain samples

Three to five micrograms of total RNA was used for cDNA synthesis by oligo (dT) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription polymerase chain reaction (RT-PCR) using SYBER/GREEN I (Applied Biosystems, Foster City, CA) was performed with an ABI PRISM 7900HT (Applied Biosystems). The comparative Ct method was used for quantification according to the manufacture's protocol (Applied Biosystems). Measurement of delta Ct was done at least in triplicate. Amplification of the single product was confirmed by monitoring the dissociation curve and by gel electrophoresis. We used two control genes (*GAPDH* and *CFL1*) for normalization. The validity of the use of *CFL1* as an internal control gene in postmortem brain samples has been shown previously [20]. Primer sequences used for the measurement of *ADAR2* are shown in Additional file 2: Table S1.

RNA editing analysis

RNA editing levels of *GRIA2*, *GRIA3*, and *GRIA4* were determined in human samples. In the case of mice, those of *Gria2*, *Gria3*, *Gria4*, *Grik1*, and *Grik2* as well as *Htr2c* were determined. The primers used for this assay are listed in Additional file 2: Table S1. Because of the limited amount of RNA samples, RNA editing analysis in human brain was performed only on the Neuropathology Consortium samples by the primer extension combined with denaturing high-performance liquid chromatography (PE-DHPLC) method, according to a previous report [42]. In brief, after RT-PCR, extension of the primer was performed before the editing site, and it was terminated by incorporation of ddNTPs. The reaction mixture was separated and quantified by denaturing HPLC using a WAVE DNA fragment analysis system with the DNASep column (Transgenomic, Hillington, United Kingdom). The gradient was prepared by mixing buffer A [0.1 M triethylammonium acetate buffer (TEAA), pH 7.0] and buffer B [25% acetonitrile in 0.1 M TEAA]. Extension products were typically eluted using a linear gradient from 18% B to 38% B. RNA editing efficiency was calculated by comparing the area of the peak corresponding to the edited and nonedited extension products.

For the mouse study, heterozygous *Adar2* knockout mice ($Adar2^{+/-}$ (n = 10) and the WT littermates (n = 10) were used for the RNA editing analysis by pyrosequencing [43]. Each group included five males and five females. Three brain areas (cerebral cortex, hippocampus, and cerebellum) were dissected from the brain of each mouse. RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). In brief, after RT-PCR with a biotinylated primer, streptavidin-sepharose beads (GE Healthcare Life Sciences, Uppsala, Sweden) and the binding buffer (10 mM Tris–HCl, 1 mM EDTA, 2 M NaCl, 0.1% Tween 20 at pH 7.6) were mixed with the RT-PCR product. The reaction mixture was placed onto a MultiScreen-HV clear plate (Millipore, Billerica, MA). After applying the vacuum, the beads were treated with a denaturation solution (0.2 N NaOH). The beads were then suspended in annealing buffer (20 mM Tris-acetate, 2 mM Mg-acetate at pH 7.6) containing a sequencing primer. The template-sequencing primer mixture was transferred onto a PSQ96 Plate (Qiagen, Venlo, Netherlands). Sequencing reactions were performed with a PSQ96 SNP Reagent Kit (Qiagen) using PSQ96MA (Qiagen) according to the manufacturer's instructions.

Behavioral analyses

Behavioral analyses were performed at BMSRC (Akashi, Japan) with $Adar2^{+/-}$ (n = 16) and the WT littermates (n = 16). All were males, aged 8–10 weeks at the initiation of the behavioral analyses. The analyses were performed in the following order: open-field test, prepulse inhibition

(PPI) test, elevated plus maze test, Morris water maze test, passive avoidance learning test, active avoidance learning test, and forced swimming test.

Open-field test

A transparent cubic box without a top (30 × 30 × 30 cm) was used. A 40-W white lamp provided room lighting, which was approximately 110 lux on the floor of the chamber. A fan attached to the upper part of the wall at one end of the chamber presented a masking noise of 45 dB. Two infrared beams were set on each wall 2 cm above the floor at intervals of 10 cm. The total number of successive interceptions of the two adjoining beams on each bank was scored as locomotion behavior. For the rearing analysis, 12 more infrared ray beams attached 4.5 cm above the floor at 2.5-cm intervals were used. The total number of vertical beam interceptions was scored as the rearing behavior. Each mouse was allowed to explore freely in the open-field area for 20 min.

Startle response and PPI test

Using a transparent acrylic box (7 × 7 × 10 cm), the startle response was detected by an accelerometer (GH-313A, Keyence, Osaka, Japan) as vibration of the box. The acoustic startle pulses of a broadband burst (115 dB, 50 ms) and tone prepulse (85 dB, 30 ms) were presented by using a speaker located in front of the box. Prepulse using light (30 ms) was also applied by a light-emitting diode (LED); however, this was not used as data because it was found that the light prepulse did not attenuate the startle response.

At the beginning of the session, 40 startle pulses were presented to test for basal startle responsiveness and its habituation. The average values of eight blocks, consisting of five startle pulses each, were used for the statistical analysis. After that, three different types of trials were performed; that is, startle pulse alone (n = 12), startle pulse preceded by a tone prepulse (n = 12), and startle pulse preceded by a light prepulse (n = 12). Prepulses were presented 50, 100, or 200 ms before the startle pulse. In total, six types of prepulse (each n = 4) were applied. The mean interval averaged 25 s (15–45 s) throughout the session. The startle response was recorded for 200 ms with a sampling frequency of 1000 Hz. The PPI test was assessed by the ratio of the mean response of the trials with one type of prepulse (n = 4) divided by the mean response of the trials without prepulse (n = 12).

Elevated plus maze test

The maze consisted of four arms (two open arms and two closed arms), 5 cm wide and 30 cm long with a gray acrylic floor, which met at a 10 × 10 cm center zone. The two closed arms had transparent walls (15 cm in height) on both sides, and the open arms had low walls (3 mm in height) on both sides. The apparatus was mounted 75 cm above the floor of the room. The room lighting was approximately 20 lux on the maze. A video camera was placed 80 cm above the maze. A fan generated a masking noise of 45 dB. The animal was placed gently onto the center of the maze and was allowed to explore the maze freely for 10 min. The number of entries into each arm and the time spent in the open arms were videotape recorded.

Morris water maze test

A round pool, with a diameter of 95 cm and depth of 21.5 cm, was placed in the center of a 140 × 130 cm room. A platform with a diameter of 11 cm was set in one of the quadrants, 5 mm beneath the surface of black water maintained at 21 ± 1°C. On the first to fifth days, five trials per day were performed for the learning phase. Each mouse was released in one of the three quadrants of the pool without the platform, and the time to reach the platform was measured. If the mouse could not reach the platform within 60 s, the experimenter placed the mouse on the platform. On the sixth day, a probe test was performed to examine whether the mouse remembered the platform's location. The mouse was released in the quadrant on the opposite side of the platform, and its behavior for 60 s was videotaped. The time the mouse stayed in the target quadrant where the platform had been placed and the immobility time were measured.

Passive avoidance learning test

A mouse was placed in a box consisting of two rooms separated by a shutter; that is, light and dark compartments (each 10 × 10 cm). In the acquisition trial, the mouse was kept in the light compartment. Five seconds later, the door to the dark compartment was opened. When the mouse moved into the dark compartment the shutter was closed and 10 s later an electrical shock (160 V, 3 s) was delivered through the grid floor. Twenty-four hours later each mouse was placed again in the light compartment and the latency to enter the dark compartment was recorded up to a maximum of 180 s.

Active avoidance learning test

The same apparatus used for passive avoidance learning was also used in the active avoidance learning test; however, there was no shutter between the light and dark compartments. The box was set in a soundproof chamber and illuminated by a 20-W white light set on the chamber. The ceiling of the dark room was made of black acryl board and the ceiling of the light room was transparent acryl board.

The training was performed for 3 days. On each day, one session consisting of 50 trials was performed. In

each trial, a condition stimulus (CS) of a 1500-Hz sound (85 dB) was followed by an unconditioned stimulus (US) of a 140-V electrical shock. The US was given 5 s after initiation of the CS and continued until the mouse escaped to the other compartment. If the mouse did not move to the other compartment, the US lasted 15 s together with the CS. If the mouse moved within 5 s after the CS, the CS was stopped and no US was given. The time from the CS to the escape and the number of escapes were used to measure the learning performance.

Forced swimming test

The animals were placed in a square pool 24 × 24 cm in size. The water temperature was maintained at 21°C. On the first day, the mice were left in the pool for 20 min and the mobility recorded during the first 5 min by the video camera was assessed. On the second day the animals were placed in the pool for 5 min and the immobility time over 5 min was recorded. The immobility was defined by two criteria: 1) no movement of the legs and the tail and 2) a completely stationary state in the pool or movement only by inertia from previous movement. The immobility was assessed from the video by three independent raters. The median value of the three raters was used for the analysis.

Pharmacological experiments

Pharmacological experiments were performed at the RIKEN Brain Science Institute. For this analysis, *Adar2*$^{+/-}$ mice (n = 34) and the WT littermates (n = 30) were analyzed. The laboratory was air-conditioned and the temperature and humidity were maintained at approximately 22–23°C and 50–55%, respectively. Food and water were freely available except during experimentation. All of the experiments were conducted in the light phase (from 9:00 a.m. to 6:00 p.m.), and the starting times of the experiments were kept constant.

For analysis of locomotor activity, an apparatus for the open-field test equipped with a small soundproof room (185 × 185 × 225 cm) was used. Each field was made of white plastic (50 × 50 × 40 cm) and illuminated by LEDs (70 lux at the center of the field). The behavior of the mouse was monitored by a charge-coupled device camera placed on the ceiling of the rack for the open fields. The distance traveled (cm) was analyzed every 5 min. Data were collected and analyzed using an Image J OF4 (O'Hara & Company, Ltd., Tokyo, Japan), which is a modified software based on the public domain U.S. National Institutes of Health (NIH) image program (developed at the NIH and at http://rsb.info.nih.gov./nih-image/).

Amphetamine was dissolved in an aqueous 0.9% NaCl solution (1 mg/mL). The stock solution was further diluted with the 0.9% NaCl solution to achieve a dose of 1.5 mg/kg in 200 μL for injection. The locomotor activity of mice was observed for 50 min before subcutaneous administration of the amphetamine solution. In the first experiment, the fold change of the activity over 50 min after the administration of amphetamine or saline compared to the activity before treatment was used as the index.

NBQX (2,3-dioxo-6-nitro-1,2,3,4- tetrahydrobenzo[f] quinoxaline-7-sulfonamide) was dissolved in a saline solution (0.9% NaCl in water) (100 mg/mL). This stock solution was further diluted with the saline solution to achieve an dose of 10 mg/kg in 200 μL for intraperitoneal injection after a 50-min adaptation period. Fifty minutes after NBQX dosing, amphetamine or saline was administered subcutaneously. The fold change of the activity from 50 min after amphetamine treatment compared to the locomotor activity during the middle 50 min after the NBQX treatment was used as an index. These two experiments used independent mice. The first experiment consisted of 11 WT and 10 *Adar2*$^{+/-}$ mice and the second experiment consisted of 19 WT and 24 *Adar2*$^{+/-}$ mice.

Statistical analyses

For the statistical analysis, two-sample *t*-test, Pearson's coefficient of correlation, one-way ANOVA, two-way ANOVA, and RMANOVA were used. For statistical analysis of behavioral battery, at first RMANOVA with factors of time and genotype was applied. When a statistically significant interaction was detected, further post hoc analysis by *t*-test was applied. The statistical analyses were performed using IBM SPSS Statistics version 20 (IBM Corporation, New York).

Additional files

Additional file 1: Figure S1. No significant effect of NBQX on locomotor activity in both WT and *Adar2*$^{+/-}$ mice. Treatment with NBQX does not suppress a locomotor activity in both WT and *Adar2*$^{+/-}$ mice.

Additional file 2: Table S1. List of primers used in this study. Forward and reverse primer sets used in RNA editing analysis and RT-PCR for human and mouse samples.

Abbreviations

AMPA: 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)-propanoic acid; *ADAR2*: Adenosine deaminase acting on RNA type 2; *HTR2C*: Serotonin 2C receptor; *GRIA2*: AMPA-type ionotropic glutamate receptor, subunit 2; *GRIA3*: AMPA-type ionotropic glutamate receptor, subunit 3; *GRIA4*: AMPA-type ionotropic glutamate receptor, subunit 4; *GRIK1*: kainate-type ionotropic glutamate receptor, subunit 1; *GRIK2*: kainate-type ionotropic glutamate receptor, subunit 2; PCR: Polymerase chain reaction.

Competing interests

The authors declare no conflict of interest regarding this work.

Authors' contribution

MKS performed behavioral and pharmacological analysis. KI and MB performed gene expression, RNA editing and behavioral analyses. TK organized the project, analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Neuron
Report

Increased L1 Retrotransposition in the Neuronal Genome in Schizophrenia

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SUMMARY

Recent studies indicate that long interspersed nuclear element-1 (L1) are mobilized in the genome of human neural progenitor cells and enhanced in Rett syndrome and ataxia telangiectasia. However, whether aberrant L1 retrotransposition occurs in mental disorders is unknown. Here, we report high L1 copy number in schizophrenia. Increased L1 was demonstrated in neurons from prefrontal cortex of patients and in induced pluripotent stem (iPS) cell-derived neurons containing 22q11 deletions. Whole-genome sequencing revealed brain-specific L1 insertion in patients localized preferentially to synapse- and schizophrenia-related genes. To study the mechanism of L1 transposition, we examined perinatal environmental risk factors for schizophrenia in animal models and observed an increased L1 copy number after immune activation by poly-I:C or epidermal growth factor. These findings suggest that hyperactive retrotransposition of L1 in neurons triggered by environmental and/or genetic risk factors may contribute to the susceptibility and pathophysiology of schizophrenia.

INTRODUCTION

Mental disorders including schizophrenia, bipolar disorder, and major depression affect a large proportion of the global population and have a major negative economic impact. Twin, family, and adoption studies indicate the complex involvement of both genetic and environmental factors for these diseases (Keshavan et al., 2011). Despite their apparent heritability, however, causative genetic factors are mostly unknown except for rare cases of schizophrenia associated with chromosomal abnormalities (Brandon and Sawa, 2011; Cook and Scherer, 2008; Karayiorgou et al., 2010). On the other hand, environmental risk factors including prenatal infection (Brown, 2006) and obstetric complications, such as neonatal hypoxia, embryonic ischemia, and gestational toxicosis (Lewis and Murray, 1987), are well-established risk factors for schizophrenia. However, it is not clarified how these environmental risk factors interact with genomic factors.

Accumulating evidence indicates that genomic DNA in the brain contains distinctive somatic genetic variations compared with nonbrain tissues (Poduri et al., 2013). These genetic signatures include brain-specific somatic mutations (Poduri et al., 2013), chromosomal aneuploidy (Rehen et al., 2005; Yurov et al., 2007), chromosomal microdeletion (Shibata et al., 2012), and the genome dynamics of nonlong terminal repeat (LTR) retrotransposons (Baillie et al., 2011; Evrony et al., 2012; Muotri and Gage, 2006). These observed somatic variations are hypothesized to contribute to the generation of functionally diversified brain cells (Muotri and Gage, 2006).

Among the known retrotransposons, only long interspersed nucleotide element-1 (L1) has autonomous retrotransposition activity. Full-length L1 elements include a 5′ UTR, two open reading frames (ORFs), and a 3′ UTR (Figure 1A). Encoded products from the ORFs contain activities required for retrotransposition and are employed in the insertion of new L1 copies as well as nonautonomous retrotransposons such as *Alu* and SVA (Cordaux and Batzer, 2009). Recent studies indicate that engineered L1 has retrotransposition activity in neural progenitor cells from rat hippocampus (Muotri et al., 2005), human fetal brain (Coufal et al., 2009), and human embryonic stem cells (Coufal et al., 2009). These in vitro findings were confirmed in human L1 transgenic mice in vivo (Muotri et al., 2005). Adult human brain cells also showed increased L1 copy number compared with nonbrain tissues (Coufal et al., 2009). Moreover, retrotransposition

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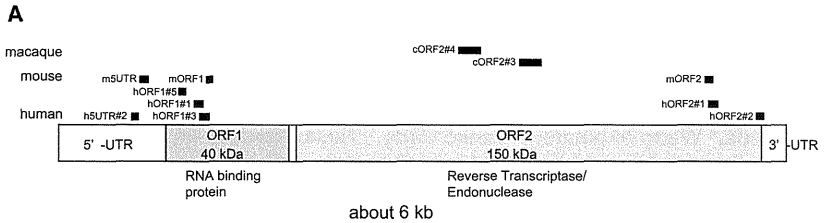
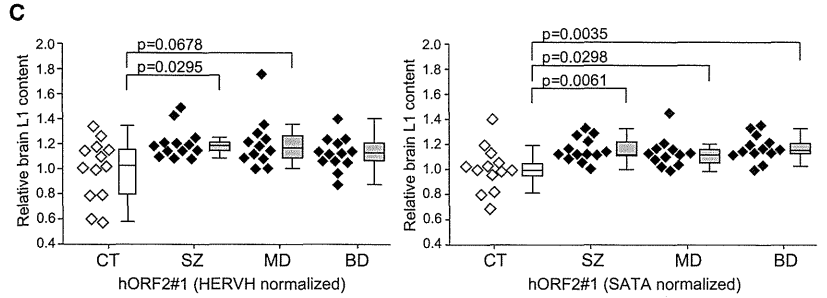
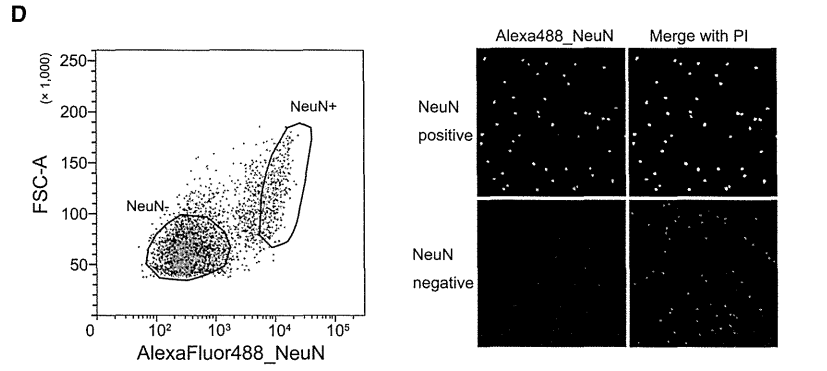


B

set	diagnosis	n	gender (F:M)	age	onset (yrs)	suicide	side (R:L)	pH	PMI (hrs)
I	CT	13	5:8	48.2±10.4	-	0	5:8	6.2±0.2	23.6±10.7
I	SZ	13	5:8	44.4±12.9	24.2±8.1	3	6:7	6.2±0.3	34.7±14.6
I	MD	12	5:7	45.2±10.0	33.5±11.8	6	3:9	6.1±0.2	27.8±10.5
I	BD	13	5:8	41.5±11.2	21.9±8.9	7	7:6	6.1±0.2	31.2±15.2
II	CT	34	8:26	44.5±7.5	-	0	18:16	6.6±0.3	29.5±13.0
II	SZ	35	9:26	42.6±8.5	21.3±6.1	7	18:17	6.5±0.2	31.4±15.3





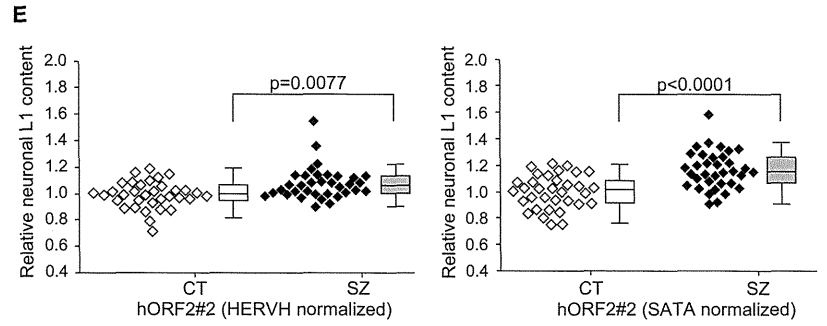


Figure 1. Increase of Brain L1 Copy Number in Schizophrenia

(A) Structure of L1 and map of the primers. Primers and probes are from previous studies (Coufal et al., 2009; Muotri et al., 2010) or designed for this study (Table S4). (B) Summary of the demographic variables of brain samples. (C) L1 copy number in set I. (D) Neuronal nuclei isolation. Left: example of NeuN-based nuclei sorting of brain cells from a patient with schizophrenia. Right: microscopic confirmation of isolated nuclei. The purity of each fraction was >95% and 99.9% for NeuN+ and NeuN−, respectively. (E) Neuronal L1 copy number in set II. In quantitative real-time PCR, L1 copy number was measured with HERVH or SATA as internal controls. The ratio of prefrontal cortex to liver (for set I) or neurons to nonneurons (for set II) was calculated and then normalized relative to the average value of control samples. Values were represented as open or closed diamonds as well as box plots. The ΔCt values of L1 and control probes were not significantly different between diagnostic groups in set I or set II. p values were determined by the Mann-Whitney U test. PMI, postmortem interval; CT, controls; SZ, schizophrenia; MD, major depression; BD, bipolar disorder; PI, propidium iodide. See also Tables S1 and S4 and Figures S1 and S2.

is active in MeCP2 mouse models and patients with Rett syndrome, indicating a role for this mechanism in this Mendelian disorder (Muotri et al., 2010). Together, these findings suggest the hypothesis that L1 retrotransposition may also be involved in the pathophysiology of mental disorders.

In this study, we quantified L1 copy number in genomic DNA derived from postmortem brains of patients with major mental disorders. We report significant increases of L1 content in the prefrontal cortex of patients with schizophrenia. To confirm this finding, we quantified L1 copy number in neurons and nonneurons from a second, independent patient cohort using NeuN-based cell sorting (Iwamoto et al., 2011; Rehen et al., 2005; Spalding et al., 2005) and found that L1 copy number in neurons was increased in patients with schizophrenia. We next quantified L1 copy number in the animal models that are known to disturb early neural development. These included maternal polyriboinosinic-polyribocytidilic acid (poly-I:C) injection in mice (Meyer and Feldon, 2012; Giovanoli et al., 2013) and chronic epidermal growth factor (EGF) injection to infant macaques (Nawa et al., 2000). We found that genomic DNA of brains from both animal models showed increased L1 copy number, addressing the importance of environmental factors during perinatal and postnatal stages. We also found that the increased L1 copy number

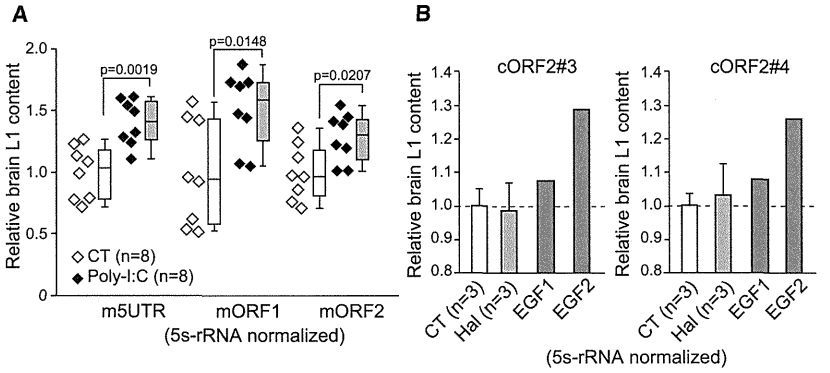


Figure 2. Increase of Brain L1 Copy Number in Animal Models

(A) Brain L1 content in the maternal poly-I:C model. p values were determined by the Mann-Whitney U test. Values were represented as open or closed diamonds as well as box plots. (B) Brain L1 content in chronic EGF or haloperidol-treated macaque models. Error bars indicate SDs. The comparative Ct method, with 5S-rRNA as an internal control, was used. The ratio of prefrontal cortex to liver (for poly-I:C model) or prefrontal gray matter to NeuN-sorted nonneurons in white matter (for macaque models) was calculated and then normalized relative to the average value of control samples. See also Table S4.

in the neurons derived from induced pluripotent stem (iPS) cells of schizophrenia patients with 22q11 deletion. The 22q11 deletion is a well-defined genetic factor and is one of the highest risk factors for schizophrenia, affecting about 1%–2% of schizophrenia patients (Karayiorgou et al., 2010). Finally, we performed whole-genome sequencing (WGS) analysis of brain and liver in controls and patients. Comparison of brain-specific L1 insertion sites revealed that brain-specific L1 insertion in patients is enriched in or near genes related to synaptic function and neuropsychiatric diseases. These results suggest that increased retrotransposition of L1 in neurons, which was triggered by genetic component and/or environmental factors at the early neural development, could contribute to the susceptibility and pathophysiology of schizophrenia.

RESULTS

Increased Brain L1 Content in Schizophrenia

We used postmortem prefrontal cortex samples of patients with schizophrenia, bipolar disorder, and major depression as well as control subjects for analysis in set I. The demographic variables are summarized in Figure 1B. We quantified L1 copy number of postmortem prefrontal cortex and liver in each subject by quantitative RT-PCR with two different internal controls, which were designed for human endogenous retrovirus (HERVH) and alpha-satellite (SATA). We found a significant increase in the brain L1ORF2 content in patients with schizophrenia (Figure 1C). A tendency toward copy number increase was also observed in mood disorders and in other L1 probes in schizophrenia (Figure S1 available online).

Somatic L1 retrotransposition was primarily found in neuronal cells (Kuwabara et al., 2009). To confirm the increased brain L1 copy number in schizophrenia and address whether this copy number increase is due to alteration of the neuronal genome, we examined an independent prefrontal cortex sample set (set II). We separated neuronal and nonneuronal nuclei from frozen brains using NeuN-based cell sorting (Figure 1D) (Iwamoto et al., 2011). NeuN is expressed in vertebrate neurons, and its antibody can be used for labeling neuronal nuclei (Mullen et al., 1992). We quantified L1ORF2 copy number of genomic DNA derived from neurons (NeuN-positive nuclei) and nonneurons (NeuN-negative nuclei) and then calculated the neuron-to-nonneuron ratio. We found a significant increase of neuronal L1ORF2 content in schizophrenia in two different internal controls (Figure 1E). The copy number of the other L1 probes tested also showed significant increase in schizophrenia compared to controls in SATA-normalized data, and similar tendency toward copy number increase was observed in HERVH-normalized data (Figure S1 and data not shown).

Assessment of Confounding Factors

We assessed the effect of confounding factors on L1 content (Table S1). Among the demographic variables tested, sample pH showed a weak correlation with L1ORF2 content in set II but not in set I. Several variables also showed weak correlations, but none showed consistency across the different internal control probes or across the two different sample sets.

To consider the possible effect of antipsychotics, we examined L1 copy number in a human neuroblastoma cell line cultured with haloperidol or risperidone for 8 days. Both antipsychotics did not modify the L1 copy number at their low or high concentrations (Figure S2). Together with the fact that the lifetime intake of antipsychotics, which was estimated as fluphenazine milligram equivalents, did not correlate with L1 copy number in both brain sets (Table S1), medication status did not affect our results.

L1 Quantification in Animal Models

To assess the potential roles of environmental factors on increased L1 copy number, we employed two different animal models that mimic environmental risk factors that affect early neural development. They included maternal poly-I:C injection in mice and chronic EGF injection to neonatal macaques. The poly-I:C, which mimics viral double-stranded RNA, injection to pregnant mice induces elevated maternal immune activation, and the offspring is known to show schizophrenia-like behavioral alterations such as impairments of prepulse inhibition and social behavior at the later stage (Meyer and Feldon, 2012). Pregnant mice received a single intraperitoneal injection of poly-I:C. L1 copy number in the prefrontal cortex of offspring was tested at postnatal day 21. We found that significant elevation of L1 copy number at all the tested probes compared to controls (Figure 2A).

We then examined the L1 copy number in macaques treated with EGF during neonatal period. Perinatal and postnatal

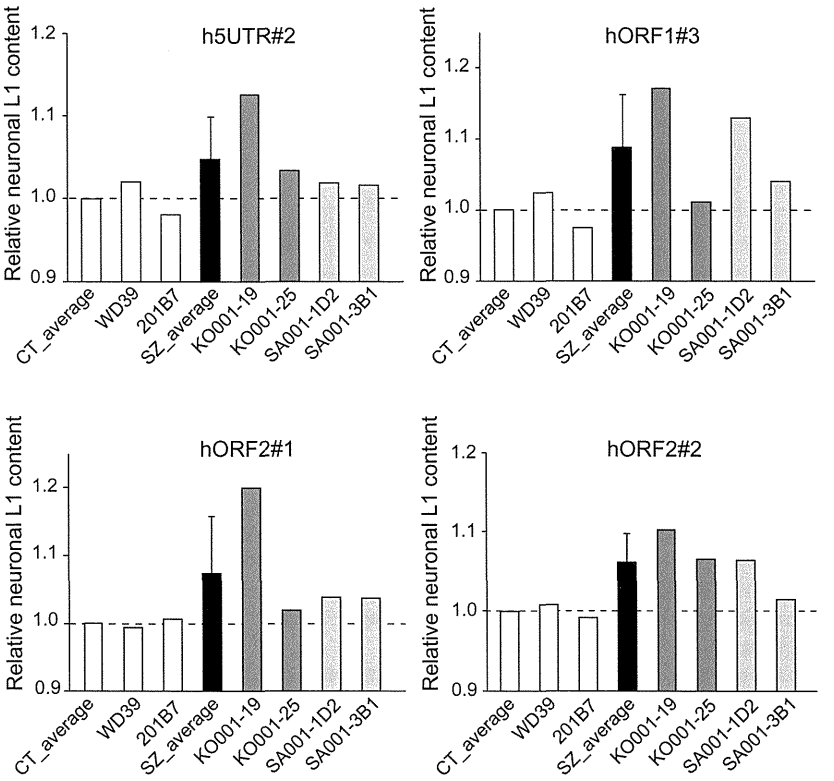


Figure 3. L1 Content in Neurons Derived from iPS Cells of Schizophrenia Patients with 22q11 Deletions
The comparative Ct method, with SATA as an internal control, was used. The ratio of NeuN-sorted neurons to nonneurons was calculated and then normalized relative to average value of control samples. Error bars indicate SDs. See also Table S4 and Figure S3.

perturbation of EGF is known to evoke schizophrenia-like phenotypes, including deficits in prepulse inhibition, latent inhibition, social interaction, and working memory, in adulthood (Nawa et al., 2009, 2000).The neonatal macaques (n = 2) subcutaneously received EGF for seven times over 11 days. After 4 and 7 years from treatment, L1 copy number in the prefrontal cortex was tested. In addition, chronic haloperidol-treated macaques (n = 3) were also tested. Due to unavailability of other tissues, we isolated nonneuronal nuclei from frozen white matter and calculated the grey matter-to-nonneuron ratio in each subject. Although statistical approach could not be applied, we observed increase of L1 copy number in EGF-treated macaques, but not in the haloperidol-treated macaques, compared to controls (Figure 2B). Taken together, these results suggest that early environmental factors play important roles in the L1 content in the brain. We further confirmed that chronic haloperidol treatment did not influence L1 copy number in this model.

L1 Quantification in the iPS Cells of Schizophrenia Patients with 22q11 Deletion

We next assessed the importance of genetic risk factor on the L1 copy number in brain. We quantified L1 copy number in the neurons derived from iPS cells of schizophrenia patients with 22q11 deletion (n = 2) as well as controls (n = 2) (Figure S3). The iPS cells were established from the fibroblasts according to the previously developed method (Imaizumi et al., 2012; Takahashi et al., 2007; M.T., unpublished data). To estimate the L1 copy number, we used two independently established iPS cell lines per patient. After induction of neuronal cells (Imaizumi et al., 2012), we isolated neuronal nuclei by NeuN-based sorting (Figure S3). We then examined L1 copy number and calculated the neuron-to-nonneuron ratio. Compared to controls, we observed consistent increase of L1 copy number in iPS cell-derived neurons of patients with schizophrenia with 22q11 deletion (Figure 3). These results suggest that the well-defined strong genetic risk factor also plays an important role in the L1 content in the brain.

Identification and Comparison of Brain-Specific L1 Transposition

We next performed WGS of brain and liver DNA from same subjects by self-assembling DNA nanoarray technology (Drmanac et al., 2010). For this experiment, schizophrenia patients (n = 3) and control subjects (n = 3) were selected to match age, PMI, gender, brain pH, and race from set I. Selected patients exhibited increased L1 content by quantitative RT-PCR assay, compared to average L1 content of the controls and selected control subjects. The WGS metrics and identified variations were summarized in Table S2. Distribution of the detected mobile elements was almost equal between the tissues and across subjects, and over the half of the identified elements was related

A

Control

insertion site	CT1	CT2	CT3	average ratio
intergenic (low)	0.64	0.76	0.69	0.69
intragenic (low)	0.36	0.24	0.31	0.31
intron (low)	0.90	1.00	0.99	0.96
exon (low)	0.10	0.00	0.01	0.04
intergenic (high)	0.65	0.81	0.69	0.72
intragenic (high)	0.35	0.19	0.31	0.28
intron (high)	0.88	1.00	0.99	0.96
exon (high)	0.12	0.00	0.01	0.04

Schizophrenia

insertion site	SZ1	SZ2	SZ3	average ratio
intergenic (low)	0.72	0.63	0.58	0.64
intragenic (low)	0.28	0.37	0.42	0.36
intron (low)	0.99	0.98	0.98	0.98
exon (low)	0.01	0.02	0.02	0.02
intergenic (high)	0.74	0.63	0.57	0.65
intragenic (high)	0.26	0.37	0.43	0.35
intron (high)	0.99	0.98	0.98	0.98
exon (high)	0.01	0.02	0.02	0.02

B

Control

Term	Count	p value
GO:0005856~cytoskeleton	74	5.92E-04
GO:0005509~calcium ion binding	56	0.0031
GO:0005930~axoneme	9	0.0095
GO:0035085~cilium axoneme	7	0.0289
GO:0003779~actin binding	26	0.0322
GO:0044425~membrane part	240	0.0387
GO:0016010~dystrophin-associated glycoprotein complex	6	0.0405

Schizophrenia

Term	Count	p value
GO:0045202~synapse	57	3.09E-09
GO:0030054~cell junction	64	8.57E-06
GO:0044459~plasma membrane part	187	1.49E-05
GO:0004674~protein serine/threonine kinase activity	58	2.27E-05
GO:0044456~synapse part	38	4.44E-05
GO:0004672~protein kinase activity	72	7.45E-05
GO:0030554~adenyl nucleotide binding	147	1.09E-04
GO:0005856~cytoskeleton	126	1.19E-04
GO:0005488~binding	820	1.25E-04
GO:0006468~protein amino acid phosphorylation	76	1.75E-04
GO:0006796~phosphate metabolic process	100	1.98E-04
GO:0006793~phosphorus metabolic process	100	1.98E-04
GO:0016773~phosphotransferase activity, alcohol group as acceptor	80	2.31E-04
GO:0001882~nucleoside binding	148	2.52E-04
GO:0005524~ATP binding	138	2.63E-04
GO:0001883~purine nucleoside binding	147	2.77E-04
GO:0032559~adenyl ribonucleotide binding	139	3.43E-04
GO:0017076~purine nucleotide binding	169	4.19E-04
GO:0000166~nucleotide binding	190	0.0011
GO:0014069~postsynaptic density	17	0.0011
GO:0032553~ribonucleotide binding	161	0.0012
GO:0032555~purine ribonucleotide binding	161	0.0012
GO:0043167~ion binding	323	0.0015
GO:0016043~cellular component organization	204	0.0018
GO:0043169~cation binding	318	0.0021
GO:0016310~phosphorylation	83	0.0021
GO:0046872~metal ion binding	315	0.0025
GO:0016301~kinase activity	85	0.0032
GO:0005737~cytoplasm	489	0.0037
GO:0008092~cytoskeletal protein binding	58	0.0045
GO:0007155~cell adhesion	74	0.0049
GO:0022610~biological adhesion	74	0.0052
GO:0019898~extrinsic to membrane	54	0.0053
GO:0043687~post-translational protein modification	108	0.0169
GO:0030030~cell projection organization	45	0.0219
GO:0005509~calcium ion binding	88	0.0234
GO:0015629~actin cytoskeleton	33	0.0439
GO:0016772~transferase activity, transferring phosphorus-containing groups	90	0.0473
GO:0045211~postsynaptic membrane	21	0.0487

C

Control

Term	Count	p value	FE
height	4	0.0132	7.7
scoliosis	3	0.0316	10.3

Schizophrenia

Term	Count	p value	FE
schizophrenia; schizoaffective disorder; bipolar disorder	5	0.0125	5.2
schizophrenia	29	0.0135	1.6
hypertension	20	0.0194	1.7
bipolar disorder	13	0.0373	1.9

Figure 4. Insertion Site, Gene Ontology, and Disease Association Analyses

(A) L1-insertion site analysis. Proportion of intergenic and intragenic L1 insertion and that of intronic and exonic L1 insertion are given. The low and high mean estimated proportions based on both less and stringent criteria are given. Note that ratios are not significantly different between patients and controls. (B) Gene ontology analysis. p values indicate Bonferroni-corrected modified Fisher's exact test p value. The terms showing $p < 0.05$ are shown for both groups. (C) Disease-association analysis. p values indicate noncorrected modified Fisher's exact test p value. FE, fold enrichment. In both analyses, gene lists generated by the stringent criteria were used. See also Tables S2 and S3 and Figure S4.

to the L1-Hs (Figure S4). Among the detected mobile element insertion sites in each sequenced sample, we first identified brain-specific L1 insertions in each subject (Tables S2 and S3). Although the total number of brain-specific L1 insertion tended to be higher in schizophrenia patients, this was not statistically significant, most likely due to the limited sample size and high interindividual variation. We then compared genomic locations of the insertion sites of brain-specific L1 between patients and controls (Figure 4A). The inter-to-intragenic L1 insertion ratio as well as exonic-to-intronic L1 insertion ratio did not differ between patients and controls. We then compared the affected genes by brain-specific L1 insertion by gene ontology approach. This analysis revealed that the number of enriched terms is higher in schizophrenia than controls, in spite that the number of brain-specific L1 insertions did not significantly differ. We found that neuronal function-related terms such as synapse and protein phosphorylation are clearly overrepresented in schizophrenia compared to controls (Figure 4B). In addition, disease-association analysis revealed that affected genes in patients are specifically enriched in terms related to schizophrenia and bipolar disorder, while those in controls are enriched in nonneuropsychiatric terms such as height and scoliosis (Figure 4C). These results were consistently confirmed when we used less stringent definition of brain-specific L1 insertion (Figure S4). In

addition, enrichment of the L1-inserted genes to the terms related to neuropsychiatric disorders in schizophrenia was also detected by the ingenuity pathway analysis (IPA) (Figure S4).

DISCUSSION

We report that the neuronal genome of schizophrenia contains higher copy number of a retrotransposon, L1. To validate this finding, we utilized iPS cells from patients with schizophrenia carrying the 22q11 deletion and observed an increase in L1 copy number in iPS cell-derived neurons. Moreover, using WGS, we found that L1 preferentially inserted into genes related to synaptic functions and schizophrenia. Animal model studies showed that environmental factors related to infection or inflammation that disturbs early neurodevelopmental processes increase L1 copy number in the brain. Collectively, these results suggest that hyperactive L1 retrotransposition into critical genes during neural development, triggered by genetic and/or environmental factors, contribute to the pathophysiology of schizophrenia. Our results significantly expand the range of neuropsychiatric illnesses linked to aberrant L1 retrotransposition, from Mendelian disease patients with *MECP2* mutations in Rett syndrome (Muotri et al., 2010) and *ATM* mutations in ataxia telangiectasia (Coufal et al., 2011) to schizophrenia, a complex mental disorder.

The observed increase of L1 content in schizophrenia was not due to, or modulated by, biological or experimental artifacts, because changes were measured in two independent patient cohorts and each result was confirmed with two different internal controls. Although the L1 region showing significant increases differed between the two brain sets, this is attributable to cohort differences amplified by the strict threshold we employed. Actually, a significant increase of L1 content was widely observed in all probes in the SATA-normalized data in set II, where neuronal L1 copy number was directly examined (Figure S1). In addition, from the data analysis utilizing lifetime intake of antipsychotics of patients, and from the cell culture and macaque experiments, we conclude that antipsychotics do not affect L1 copy number in the brain. A significant increase was also observed in patients with mood disorders in one internal control in set I (Figure 1C). Future work will clarify whether there are L1 content increases in other mental disorders using larger and/or stratified patient cohorts.

L1 retrotransposition has been detected during adult neurogenesis in the rat hippocampus, indicating that neural progenitor cells retain retrotransposition activity even in adult stages (Muotri et al., 2009). However, we analyzed potential confounding factors, including age, age of onset, and duration of illness, and did not observe any significant correlation with L1 copy number in the brain. The transcript level of L1 in adult brain sample was also increased in patients compared to controls (data not shown). However, elevated expression is unlikely to contribute to increase of L1 copy number in patients, as significant increase of L1 transcripts was detected only in the 5′ region of L1 such as 5′ UTR and ORF1. These results suggest that L1 copy number does not globally increase with aging and that the variation of L1 copy number in patients is probably confined to early neurodevelopmental stages, at least in the prefrontal cortex. This prediction would be consistent with the neurodevelopmental hypothesis of schizophrenia, where abnormalities during critical early periods of brain development may trigger the later appearance of clinical symptoms (Bloom, 1993; Murray et al., 1992; Weinberger, 1987).

In Rett syndrome, increased L1 copy number in human brain was linked to mutations in *MECP2* (Muotri et al., 2010) and *MeCP2* knockout mice also showed increased L1 content (Muotri et al., 2010). It has also been suggested that SOX2 and MECP2 regulate L1 transcription in neurons (Muotri et al., 2005; Yu et al., 2001). However, we did not observe a significant correlation between *MECP2* or *SOX2* expression and brain L1 content, by using the previously performed gene expression analyses on the same sample sets (Iwamoto et al., 2004, 2005) (data not shown). In addition, patients with high levels of L1 copy number (two schizophrenia and one major depression in set I, and two schizophrenia patients in set II) did not show altered *MECP2* or *SOX2* expression levels (data not shown). These findings suggest that the molecular mechanism of increased L1 in schizophrenia is different from Rett syndrome.

In this study, we found that both early environmental and well-defined strong genetic factors for schizophrenia are involved in the increase of L1 copy number in the brain. A recent study using the poly-I:C model indicated that the offspring of this model had exacerbated schizophrenia-like phenotypes, if they were exposed to environmental stress during puberty, suggesting that early environmental factors can lower the threshold for onset of schizophrenia (Giovanoli et al., 2013). Therefore, increased L1 insertions induced by environmental factors may increase the susceptibility to schizophrenia by disrupting synaptic and schizophrenia-related genes in neurons, rather than being a direct cause of the disease. On the other hand, the pathological consequences of increased L1 content in neurons derived from iPS cells of schizophrenia patients with 22q11 deletions remain unclear. We chose patients with 22q11 deletions to examine L1 dynamics where there is a well-defined strong genetic risk for schizophrenia. In *MeCP2*-knockout mice, Rett-like behavioral abnormalities could be rescued by the re-expression of wild-type MeCP2 at both young and adult stages (Cobb et al., 2010; Ehninger et al., 2008), suggesting that L1 content itself may not be directly causal to disease phenotypes but instead modulate phenotypic variability among patients (Muotri et al., 2010). Similarly, we speculate that the L1 increase in schizophrenia patients with 22q11 deletions is likely to modulate phenotypes of schizophrenia rather than a direct cause, because many genes related to schizophrenia, such as *TBX-1*, *SEPT5*, *COMT*, and *PRODH*, are located within the deletion (Hiroi et al., 2013; Karayiorgou et al., 2010). Nevertheless, our findings will facilitate further studies of the mechanism of increased L1 retrotransposition associated with schizophrenia.

Our WGS analysis could not detect increased brain-specific L1 insertions in schizophrenia; however, we found that L1 insertions were more frequent in genes for synaptic function and schizophrenia relative to controls. Evrony et al. cloned one L1 insertion event from 300 single neurons and showed that 2 of 83 cortical neurons from an individual had this insertion, but detection of such a low level mosaic insertion in bulk brain tissue of the same individual was difficult and needed optimization

(Evrony et al., 2012). Thus, rare L1 insertion events could be missed in our WGS analysis. Apart from L1, nonautonomous retrotransposons such as *Alu* and SVA also show an increased copy number in the brain, possibly via the aid of L1 ORF products (Baillie et al., 2011) and their copy number might also be increased in patients. Further studies on the neuronal genome of patients with mental disorders, and supporting mechanistic evidence from animal and cellular models, may establish a broader role for instability of neural genome in the pathophysiology of schizophrenia. We expect that our findings will promote the further study of genomic instability in disease etiology due to L1 retrotransposition in brain development.

EXPERIMENTAL PROCEDURES

Postmortem Samples

Postmortem brain and liver samples were obtained from the Stanley Medical Research Institute. The demographics are summarized in Figure 1B and are described at the web site (http://www.stanleyresearch.org/). Ethics committees of RIKEN and the University of Tokyo Faculty of Medicine approved the study.

Animal Models

Animal experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and guidelines of relevant facilities. For poly I:C model, pregnant mice (C57BL/6) received either a single intraperitoneal injection of poly-I:C (2 mg/ml, Sigma-Aldrich) dissolved in PBS (20 mg/kg) or an equivalent volume of PBS at embryonic day 9.5. At postnatal day 21, tissues were dissected from pups. For macaque models, cynomologus monkeys (*Macaca fascicularis*) (4 years old; all males) were given oral haloperidol (0.25–0.5 mg/kg; Wako Pure Chemical Industries) or vehicle for 2 months (Shibuya et al., 2010). After transiently separating two male monkey neonates (2 weeks old) from dams, neonates received subcutaneous administration of human recombinant EGF (0.3 mg/kg, Funakoshi) for seven times over 11 days and then quickly returned to their dams. Preliminary behavioral assessment of the EGF-treated monkeys was performed at ages of 4 and 6 years and reported (Nawa et al., 2009). These monkeys were sacrificed at the age of 4 and 7 years with the overdose of pentobarbital (26 mg/kg; 65 mg/ml). Experiments were subjected to review by the Ethical Committee of Shinn Nippon Biomedical Lab.

iPS Cells

All procedures for skin biopsy and iPS cell production were approved by the Keio University School of Medicine ethics committee and RIKEN ethics committee. The 201B7 iPS cells were kindly provided by Dr. Yamanaka (Takahashi et al., 2007). For the control WD39, a skin-punch biopsy from a healthy 16-year-old Japanese female obtained after written informed consent was used to generate iPS cells (Imaizumi et al., 2012). 22q11.2 deletion syndrome iPS cells (SA001 and KO001) were generated from a 37-year-old Japanese female patient (Toyosima et al., 2011) and a 30-year-old Japanese female patient, respectively, using the same method used to generate the WD39 (M.T., unpublished data). 22q11 deletion was characterized by the CGH array analysis (Figure S3). Production and maintenance of iPS cells were performed according to the previous studies (Imaizumi et al., 2012; Takahashi et al., 2007). All the iPS cells and differentiated neuronal cell lines were characterized with immunofluorescence staining and their morphologies (Figure S3).

L1 Copy Number Estimation

We performed either Taqman-based quantitative real-time PCR according to Coufal et al. (2009) with minor modifications (100 or 500 pg DNA as starting material and single amplicon analysis) or SYBR-Green-based quantitative real-time PCR according to Muotri et al. (2010). SYBR-Green assay was performed using 500 pg DNA and Power SYBR Green PCR Master Mix (Life Technologies). Primers, probe location, and reaction chemistry are listed in Figure 1A and Table S4. Quantification was performed in triplicate. A nonparametric Mann-Whitney U test was employed for two group comparison and $p < 0.05$ was considered significant.

Whole-Genome Sequencing

WGS of brain and liver samples from controls and schizophrenia patients was performed by Complete Genomics, with the paired-end library preparation and sequencing-by-ligation using self-assembling DNA nanoball (DNB) (Drmanac et al., 2010). Data process, mapping, and detection of variations were performed using the software developed by the Complete Genomics (version 2.2.0.26 and format version 2.2). Among the detected mobile insertion elements, we compared the genomic location of L1 insertion between brain and liver within an individual and identified brain-specific L1 insertions.

Further experimental details are available in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.10.053.

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Comprehensive survey of CNVs influencing gene expression in the human brain and its implications for pathophysiology☆

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ABSTRACT

Copy number variations (CNVs) contribute to neuropsychiatric diseases, which may be partly mediated by their effects on gene expression. However, few studies have assessed the influence of CNVs on gene expression in the brain. The objective was to perform an unbiased comprehensive survey of influence of CNVs on gene expression in human brain tissues. CNV regions (CNVRs) were identified in 72 individuals (23 schizophrenia, 23 bipolar disorder and 26 controls). Significant associations between the CNVRs and gene expression levels were observed for 583 CNVR-expression probe pairs (293 unique eCNVRs and 429 unique transcripts), after corrections for multiple testing and controlling the effect of the number of subjects with CNVRs by label swapping permutations. These CNVRs affecting gene expression (eCNVRs) were significantly enriched for rare/low frequency ($p = 1.087 \times 10^{-10}$) and gene-harboring CNVRs ($p = 1.4 \times 10^{-6}$). Transcripts overlapping CNVRs were significantly enriched for glutathione metabolism and oxidative stress only for cases but not for controls. Moreover, 72 (24.6%) of eCNVRs were located within the chromosomal aberration regions implicated in psychiatric-disorders: 16p11.2, 1q21.1, 22q11.2, 3q29, 15q11.2, 17q12 and 16p13.1. These results shed light on the mechanism of how CNVs confer a risk for psychiatric disorders.

1. Introduction

A key objective in genetic research is to link genomic variation to phenotype differences to uncover normal as well as pathological variation. The influence of single nucleotide polymorphisms on phenotypic variation has been extensively studied; however, it is only recently that other DNA alterations such as copy number variations are being investigated. Copy number variations (CNVs) are DNA segments present at variable copy numbers and owing to their large size, contribute to a substantial proportion of the variation in the human genome (Ionita-Laza et al., 2009; Redon et al., 2006). Among the CNVs, rare CNVs are of more interest because they are presumably enriched in de novo events. Under the rare-variant common disease hypothesis, multiple rare variants with high effect sizes in aggregation, contribute substantially to the illness, hence these rare variants are of great interest since they have not been subject to selection as yet (Zhang et al., 2009). Rare copy number variations have been reported in individuals with neurological and psychiatric disorders such as schizophrenia (International Schizophrenia Consortium, 2008; Levinson et al., 2011; McCarthy et al., 2009), autism (Hedges et al., 2012), bipolar disorder (Zhang et al., 2009) and mental retardation (Guilmatre et al., 2009).

Although a large number of CNVs have been identified in a variety of different species and range of diseases, the functional impact of CNVs at the molecular level remains largely unexplored. One way to assess the functional impact of copy number variations is via its

Abbreviations: CNVs, copy number variations; LCLs, lymphoblastoid cell lines; CNVRs, copy number variable regions; eCNVRs, expression influencing copy number variable regions; kb, kilo base pairs; Mb, mega base pairs; fdr, false discovery rate.

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