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January 21, 2014

ICOC Review Committee

Dear ICOC Programmatic Review Committee:

Thank you for the reviews of our application for the **Center of Excellence for Stem Cell Genomics**. We are very pleased with the overall enthusiasm for our Genomics Center and the Data Coordination and Management Center. We would like to comment on two of the Center-Initiated Projects, CIP2 and CIP3 that had concerns raised, and why we strongly feel that it would be advantageous to include in our center.

CIP-2
We thank the CIRM review committee for their careful reading of the proposal and their evaluation. We were gratified to see that the committee was able to reach a consensus conclusion that CIP2 "is a technological tour-de-force, exploiting highly innovative, cutting edge techniques that are unparalleled around the world," and that "reviewers judged this team uniquely qualified to undertake a project of such magnitude and ambition. The project leadership is the key strength of this application, representing a powerful merging of expertise in technology development, cancer biology, and computational modeling."

Despite these statements of support, the committee was split on support for CIP2, with a faction voting to remove it from the center. In this letter we address the objections of the negative faction of the committee, which are based on material errors of fact or scientific details that were not explicitly addressed in the proposal due to space limits. In doing so, we hope to convince the committee that the programmatic benefits of CIP2 far outweigh the financial risks. We note that after the proposal was submitted, the core technology of CIP2, single cell genomics, was named "Method of the Year" in 2013 by Nature Methods, who also recognized CIP2 PI Stephen Quake as a pioneer of the field. Funding CIP2 will make these cutting edge technologies available to the larger stem cell community in California via the collaborative project mechanism; conversely, if CIP2 is deleted researchers in California will be denied access to a fundamental, cutting edge genomic technology in their CIRM Genomics Center.

We would like to emphasize that this CIP will be a crucial resource for CIRM and the people of the state of California. One of the central goals of CIRM is to use pluripotent stem cells, either embryonic stem cells (ESCs) or induced-pluripotent stem cells (iPSCs), to develop cell based therapies for regenerative medicine. To do this, it is critical that the ESCs/iPSCs-derived cell products used for transplantation have been correctly programmed into the proper cell type. Improper reprogramming of ESCs/iPSCs into malignant or pre-malignant cells is a widely documented problem. Use of such cells for organ repair/regeneration would be devastating. As noted above by the referee comments, the leadership of this team is uniquely qualified to tackle this problem. Indeed, the principle investigators of CIP2 have a long history of collaboration to develop the tools and strategies needed to identify correctly and incorrectly reprogrammed cells. Integration of the tools, techniques and data described in this CIP will improve most, if not all, clinical studies using transplantation of cells derived from either ESCs or iPSCs.

Neuron
Report

Increased L1 Retrotransposition in the Neuronal Genome in Schizophrenia

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http://dx.doi.org/10.1016/j.neuron.2013.10.053

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.

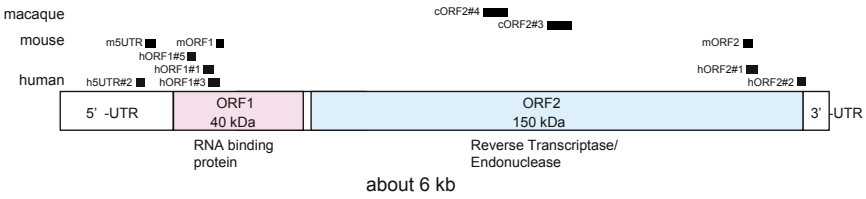
INTRODUCTION

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

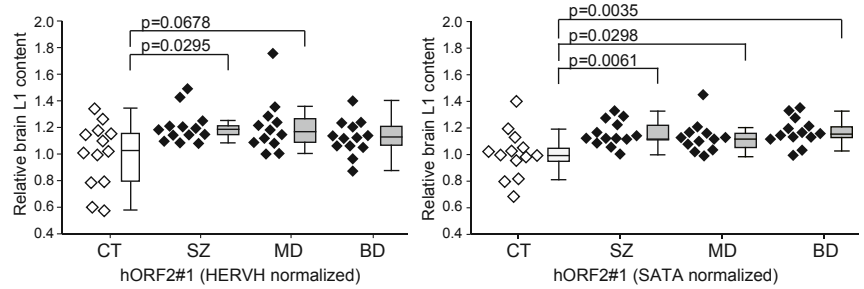
A



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

C



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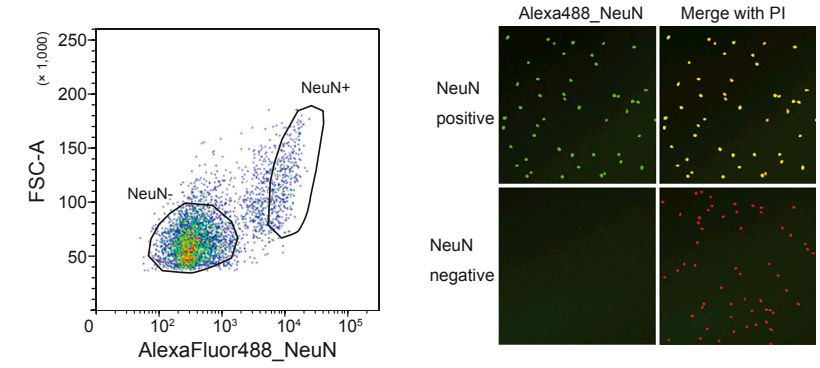

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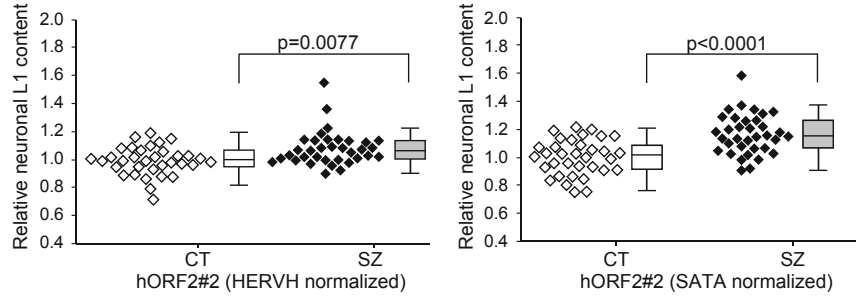

Figure 1. Increase of Brain L1 Copy Number in Schizophrenia

(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

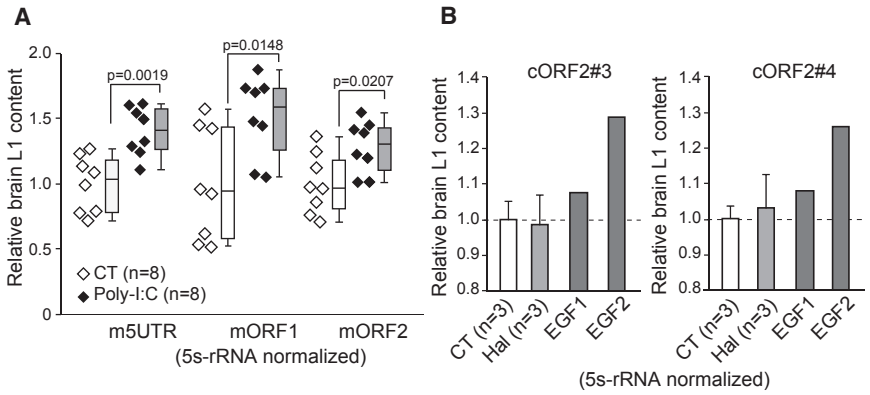

Figure 2. Increase of Brain L1 Copy Number in Animal Models

(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

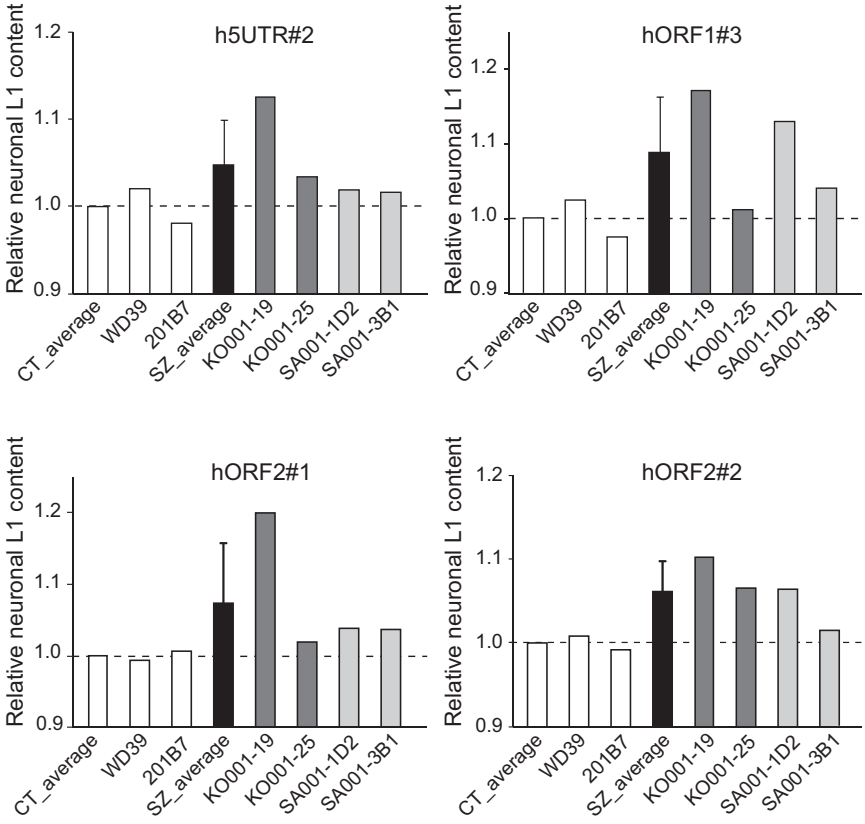

Figure 3. L1 Content in Neurons Derived from iPS Cells of Schizophrenia Patients with 22q11 Deletions

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.

ACKNOWLEDGMENTS

This work was supported in part by the Grant-in-Aid for Scientific Research on Innovative Areas (Unraveling the microendophenotypes of psychiatric disorders at the molecular, cellular, and circuit levels) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to T.Y., H.N., T.K., and K.I., and a Grant-in-Aid from Ministry of Health, Labour and Welfare to T.K. This work was also supported by JST, CREST to T.K. and by JST, PRESTO to K.I. This work was also supported in part by Leading Project for Realization of Regenerative Medicine from MEXT and "Funding Program for World-Leading Innovative R&D on Science and Technology" to H.O., and by the "Development of biomarker candidates for social behavior" carried out under the Strategic Research Program for Brain Sciences from MEXT to T.Y. and K.K. This work was also supported in part by the Collaborative Research Project of the Brain Research Institute, Niigata University. Postmortem samples were donated by the Stanley Medical Research Institute, courtesy of Drs. Michael B. Knable, E. Fuller Torrey, Maree J. Webster, and Robert H. Yolken. We thank Tomoko Toyota and Atsuko Komori-Kokubo at RIKEN BSI for their technical assistance. We also thank Kenji Ohtawa at Research Resources Center at the RIKEN BSI for the cell-sorting analysis. M.B., F.S., and K.I. belong to the Department of Molecular Psychiatry, which is endowed by Dainippon Sumitomo Pharma and Yoshitomiyakuhin. H.O. is a scientific consultant for San Bio, Eisai, and Daiichi Sankyo. T.K. received a grant from Takeda Pharmaceutical. These companies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Accepted: October 18, 2013
Published: January 2, 2014

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The reviewers wanted more details about which cell phenotypes would be isolated for study and whether meaningful conclusions could be drawn in the absence of functional definition. The specific aim of the proposal is in fact to create a genomic resource of cell types; the goal is to discover new cell types and not to isolate already well-studied cells. The reviewers addressed similar questions specifically to the pancreas and asserted a lack of familiarity with the biology of the pancreas; we note that our collaborator Michael Clarke co-discovered the first pancreatic cancer stem cells (Li et al, "Identification of Pancreatic Cancer Stem Cells," Cancer Research 2007) and continues to have pancreatic cancer organoids and cell lines propagating in his laboratory. With respect to the Down syndrome aims, the reviewers had reservations that "key aspects of Down Syndrome brain pathology develop prior to the time frame in which the specimens for analysis are to be collected." While it is true that the final defect is in late gestation, one can utilize human fetal neural progenitor cells in culture and these cells have a defect *in vitro* culture that can be measured. (Bhattacharyya et al "A Critical Period in Cortical Interneuron Neurogenesis in Down Syndrome Revealed by Human Neural Progenitor Cells," Developmental Neuroscience 2009). We note that our collaborator Michael Clarke's work in the space comes up in the top two hits when googling "Down syndrome stem cell", and that Stephen Quake's work in this area has led to the first non-invasive prenatal diagnostic for Down syndrome.

In conclusion, we point out that it is rare to find scientific consensus on ambitious, revolutionary projects; indeed, the human genome project was quite controversial when it was launched and was only supported by a minority in the community. It wasn't until years later that the full impact was appreciated by the broader scientific community. CIP2 is of a similar flavor, and CIRM is in a unique position to fund cutting edge science at the interface of genomics and stem cell analysis while bringing the most novel techniques to the larger stem cell community in California. It will provide a strategy for identifying correctly reprogrammed cells for cell based regenerative medicine. The brain and pancreas were chosen as model systems because cell based therapies for neurodegenerative diseases and diabetes are two of the most important indications for such cell based therapies.

CIP-3

The reviewers recommended removal of CIP-3 from the center due to concerns regarding feasibility of using an iPSC-genomics based approach to study schizophrenia. As outlined below we disagree with the reviewers' comments on several fronts. However there are also programmatic reasons to retain this project as part of the Center of Excellence in Stem Cell Genomics: First, psychiatric disease is a major burden of disease in California and it is important to have a research program focused on diseases of the nervous system as part of the overall CIRM Genomics Center. Second, this center-initiated project focuses on using state-of-the-art analysis of the epigenome to study neurons derived from human iPSC. Excision of CIP-3 will remove epigenomics as a major research platform within the center research program. Retention of this project in the Center brings synergies to the research team and the overall program on both of these fronts.

Two main issues were raised in the reviewer comments. As a material dispute of fact, we disagree with both. The first, considered a "fundamental flaw", was that the proposed analysis of twins discordant for schizophrenia was not powered adequately. A power analysis for any study requires some evidence or prediction about the variance and ability to detect differences between groups based on published literature. In this case we have published results (Brennand et al, Nature 473:221-225, 2011) demonstrating that with 4 controls and 4 sporadic schizophrenic iPSC-derived neurons statistically significant differences are readably detected both functionally, and in microarrays, that can be repeated in multiple assays. Several additional

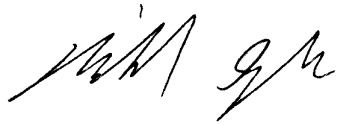
studies and reviews have highlighted the value of iPS cells and reveal that the small sample size is not an issue when you are examining the neurons derived from patient cells. A good example is a recent manuscript in Neuron showing the ability to detect significant increases in L1 insertions in genomes of iPSC-derived neurons from schizophrenic patients that could be confirmed in brain tissue (Bundo et al, Neuron, January 2014, attached) – these exciting and definitive findings were found using iPSC from three subjects. Our twin cohort also includes three schizophrenic subjects. Based on the high penetrance of phenotypes seen in both the Brennand and Bundo studies, our analysis will produce similar significant results.

One potential point of misunderstanding is the difference between case studies or small sample studies that are geared toward detailed ENDOPHENOTYPING – using in vitro cell samples and detailed physiological measurements of dynamic cell function combined with comprehensive genomic analysis of the cells being evaluated – vs. Human genome-wide association studies (GWAS) that correlate behavior phenotypes with genomic analyses from blood samples from the patients. Members of the review panel included experts in using GWAS for schizophrenia. However, the differences in these approaches are profound not only because of the level of analysis, but also with the cell-based assays mechanisms can be explored: genetic pathways that are identified as being of interest can be tested by manipulating the genes and networks experimentally and proving whether those genes are important in the neurons derived from the patients and controls. This level of analysis and confirmation of "hits" is not possible in the GWAS approach. In the current proposal we not only have a cohort of 12 twins, but as stated in the application we have available information from all of the sporadic cases of schizophrenia that we have already published. It must be emphasized that this is a STEM CELL approach, not a GWAS analysis.

The reviewers were also concerned that iPSC model studies will not reflect the biology of the brain. While iPSC technology is still relatively new, we and others have made many fundamental discoveries using iPSCs and iPSC-derived neuronal cells to recapitulate in a dish many of the phenotypes associated with neurons isolated from diseased and normal brain tissue. This breakthrough technology is already changing the way human disease is studied and new therapies are being developed, many with funding from CIRM. In the 2011 Nature article noted above we demonstrated reliable phenotypes associated with iPSC-derived neurons from patients with schizophrenia. The purpose of CIP-3 is to use iPSC approaches in a controlled genome environment (twins) with epigenetic analysis to learn more about the drivers for these unique phenotypes. With regard to issues related to the incomplete epigenetic landscape during reprogramming, we have obtained full genome sequencing at 40X depth all 12 subjects used in this study that will serve as a reference to identify any genomic changes that occur through reprogramming. Significant published evidence using iPS cells, including several studies from the Ecker and Ren laboratories, makes it clear that if there are epigenetic variations that do occur due to reprogramming they can be detected easily (Lister et al. 472:68-73, 2011).

We hope that you will support the inclusion of these projects in our center.

Sincerely,



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