ONLINE FIRST

Differences in the Circuitry-Based Association of Copy Numbers and Gene Expression Between the Hippocampi of Patients With Schizophrenia and the Hippocampi of Patients With Bipolar Disorder

Guoqing Sheng, PhD; Matthew Demers; Sivan Subburaju, PhD; Francine M. Benes, MD, PhD

Context: *GAD67* regulation involves a network of genes implicated in schizophrenia and bipolar disorder. We have studied the copy number intensities of these genes in specific hippocampal subregions to clarify whether abnormalities of genomic integrity covary with gene expression in a circuitry-based manner.

Objective: To compare the copy number intensities of genes associated with *GAD67* regulation in the stratum oriens of sectors CA3/2 and CA1 in patients with schizophrenia, patients with bipolar disorder, and healthy controls.

Design: Samples of sectors CA3/2 and CA1 were obtained from patients with schizophrenia, patients with bipolar disorder, and healthy controls. Genomic integrity was analyzed using microarrays, and the copy number intensities identified were correlated with the gene expression profile from a subset of these cases previously reported.

Setting: Harvard Brain Tissue Resource Center at McLean Hospital, Belmont, Massachusetts.

Patients: A total of 15 patients with schizophrenia, 15 patients with bipolar disorder, and 15 healthy controls.

Main Outcome Measures: The copy number intensities for 28 target genes were individually examined using single-nucleotide polymorphism microarrays and correlated with homologous messenger RNA (mRNA) fold changes.

Results: The copy number intensities examined using both microarrays and quantitative real-time polymerase chain reaction for the *GAD67* gene were significantly decreased in sector CA3/2 of patients with schizophrenia and patients with bipolar disorder. Other genes associated with *GAD67* regulation also showed changes in copy number intensities, and these changes were similar in magnitude and direction to those previously reported for mRNA fold changes in sector CA3/2 but not sector CA1. Moreover, the copy number intensities and mRNA fold changes were significantly correlated for both patients with schizophrenia ($r=0.649$; $P=.0003$) and patients with bipolar disorder ($r=0.772$; $P=.0002$) in sector CA3/2 but not in sector CA1.

Conclusion: Insertions and deletions of genomic DNA in γ-aminobutyric acid cells at a key locus of the hippocampal circuit are reflected in transcriptional changes in *GAD67* regulation that are circuitry-based and diagnosis-specific.

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Author Affiliations: Program in Structural and Molecular Neuroscience, McLean Hospital, Belmont (Drs Sheng, Subburaju, and Benes and Mr Demers), and Program in Neuroscience (Dr Benes) and Department of Psychiatry (Drs Sheng, Subburaju, and Benes), Harvard Medical School, Boston, Massachusetts.

COPY NUMBER ABNORMALITIES have been found in genomic material from patients with many different brain disorders.[1-11] However, their significance for understanding the heritability and/or pathophysiology of such illnesses is not understood. The present study has attempted to explore this question by analyzing copy number intensities in laser-microdissected samples of the hippocampus from patients with schizophrenia and patients with bipolar disorder. In both disorders, dysfunction of γ-aminobutyric acid (GABA)–ergic cells has been inferred from the finding of decreased expression of the glutamic acid decarboxylase 67 kD isoform (GAD67), a key marker for GABAergic function.[12-15] There is a network of genes associated with the regulation of *GAD67*.[16] This network appears to interact with other functional gene clusters, such as neurogenesis, cell cycle regulation, and the DNA damage response.[17] Together, the latter 3 clusters play a critical role in maintaining genomic integrity, by detecting and repairing insertions, deletions, or perhaps even sequence rearrangements. If not repaired, such changes could theoretically result in the abnormal transcriptional regulation of genes associated with the regulation of *GAD67*.

In our study, samples have been obtained from the stratum oriens of sectors CA3/2 and CA1 (eFigure 1, http://www.archgenpsychiatry.com), a layer of the hippocampus in which GABA cells are the exclusive neuronal cell type. A preponderance of postmortem abnormalities in patients with schizophrenia and in patients with bipolar disorder have been found in sector CA3/2.[18,19] These samples have been subjected to a microarray-based analysis of copy number intensities for 28 candidate genes involved in *GAD67* regulation, neurogenesis, cell cycle regulation, and the DNA damage response. The results presented demonstrate that there are highly significant changes in the magnitude and direction of copy number intensities for specific target genes associated with *GAD67* regulation in patients with schizophrenia and patients with bipolar disorder. Additionally, these copy number intensities show parallel variations in the expression of the respective messenger RNAs (mRNAs) for these target genes. To our knowledge, this is the first demonstration that disturbances in genomic integrity may contribute to GABA cell dysfunction in schizophrenia and bipolar disorder. Changes in copy number intensities vary in accordance with the disturbances in the expression of mRNAs associated with *GAD67* regulation, but the patterns seen are fundamentally different for patients with schizophrenia and patients with bipolar disorder, suggesting that these changes occur in a circuitry-based and diagnosis-specific manner.

METHODS

PARTICIPANTS

For the analyses of copy number intensities, postmortem samples of hippocampi from 15 healthy controls, 15 patients with schizophrenia, and 15 patients with bipolar disorder were obtained from the Harvard Brain Tissue Resource Center at McLean Hospital in Belmont, Massachusetts. The cases were group-matched for age, postmortem disorder, hemisphere, sex, and tissue pH (eTable). A subset of these cases (7 in each group) were described in an earlier microarray-based study,[16] and the data from these cases were used to associate gene expression changes for the target genes with the respective copy number intensities obtained in the present study. Psychiatric diagnoses were established using a retrospective review of medical records and an extensive family questionnaire that includes information about medical history, psychiatric condition, and social demographics. The diagnosis of schizophrenia was made using the criteria of Feighner et al,[20] whereas the diagnosis of bipolar disorder was made according to *DSM-III-R* criteria.

TISSUE PREPARATION

Sections (20 µm) were cut from a paraffin block using with a Jung RM2025 microtome and mounted on Leica frame slides. The sections were then deparaffinized and rehydrated in a graded series of xylene and ethanol. Nissl-stained sections were examined microscopically to ensure that each was cut in a transverse plane through the hippocampus and that all of the typical cytoarchitectonic features were present. A laser-microdissected apparatus was used to sample the stratum oriens of sectors CA2/3 and CA1. Because of the hypothesis-driven design of our study, we chose to contrast the findings in the stratum oriens of CA3/2 with those of sector CA1, which is also a layer in which GABA cells are the exclusive neuronal cell type.[21] This layer in CA1 is, however, uniquely different from its homologue in CA3/2 in terms of its cytoarchitectonic detail, connectivity,[22] functional integration,[23] and gene expression patterns.[17,21,24] For these reasons, we chose the stratum oriens of sector CA1 as a comparison site for changes in genomic integrity and mRNA expression in hippocampal GABA cells. There are approximately 3 times as many glial nuclei than neuronal nuclei in the stratum oriens of both sectors. Neurons are readily distinguishable from glia by their Nissl-positive cytoplasm and dendrites, their euchromatin content, and the size of their cell bodies, which are typically much larger. Glial nuclei show a predominance of heterochromatin, which is associated with gene silencing.[25] These observations, together with our in situ hybridization studies of this layer, have suggested that very little gene expression is occurring in the glial cells at this locus.

DNA EXTRACTION AND ARRAY HYBRIDIZATION

A QIAamp DNA FFPE Tissue Kit (Qiagen) was used to extract genomic DNA from formalin-fixed, paraffin-embedded tissue. After extraction, the whole genome was amplified with a REPLI-g kit (Qiagen). DNA digestion, labeling, and hybridization were performed according to the manufacturer's instructions. In brief, genomic DNA (500 ng) is digested with *Nsp*I and *Sty*I restriction enzymes and ligated to adaptors that recognize the cohesive 4–base pair (bp) overhangs. All fragments resulting from restriction enzyme digestion were substrates for adaptor ligation. A generic primer that recognizes the adaptor sequence was used to amplify adaptor-ligated DNA fragments. Polymerase chain reaction (PCR) conditions were optimized to preferentially amplify fragments in the 200- to 1100-bp size range. The PCR amplification products for each restriction enzyme digest were combined and purified using polystyrene beads. The amplified DNA was then fragmented, labeled, and hybridized to Affymetrix Genome-Wide Human SNP Array 6.0. After the arrays were washed and stained, the copy number intensities were analyzed.

DATA ANALYSES

The scanned images of single-nucleotide polymorphism (SNP) arrays were analyzed using the Affymetrix Genotyping Console 2.0 and the Affymetrix Genotyping Tools software. Although copy number variants are commonly used as an end point in genetic studies of large populations or in searches for risk genes, copy number intensities provide greater sensitivity and more robust findings than the rounded-off ratios that result from analyses of copy number variants. The copy number intensities were determined according to the hybridization intensity data generated from each SNP probe using dChip software.[26] To calculate mean copy number values for selected regions, the output of the SNP probe intensities were exported to Excel (Microsoft). Copy number intensity changes were measured by comparing the hybridization intensities of healthy controls with those of the patients with schizophrenia or bipolar disorder.

VERIFICATION OF GENE COPY NUMBERS BY QUANTITATIVE REAL-TIME PCR

To validate copy number intensity changes identified by SNP arrays, we performed quantitative real-time PCR for *GAD67* using genomic DNA (gDNA) from 6 controls, 6 patients with schizophrenia, and 6 patients with bipolar disorder. *GAD67* gene probes was purchased from Applied Biosystems (the probes' identification numbers are Hs 01242242_cn and Hs 01602806_cn). Each 20-µL assay contained 10 ng of gDNA, 900nM each of forward and reverse primers for the reference

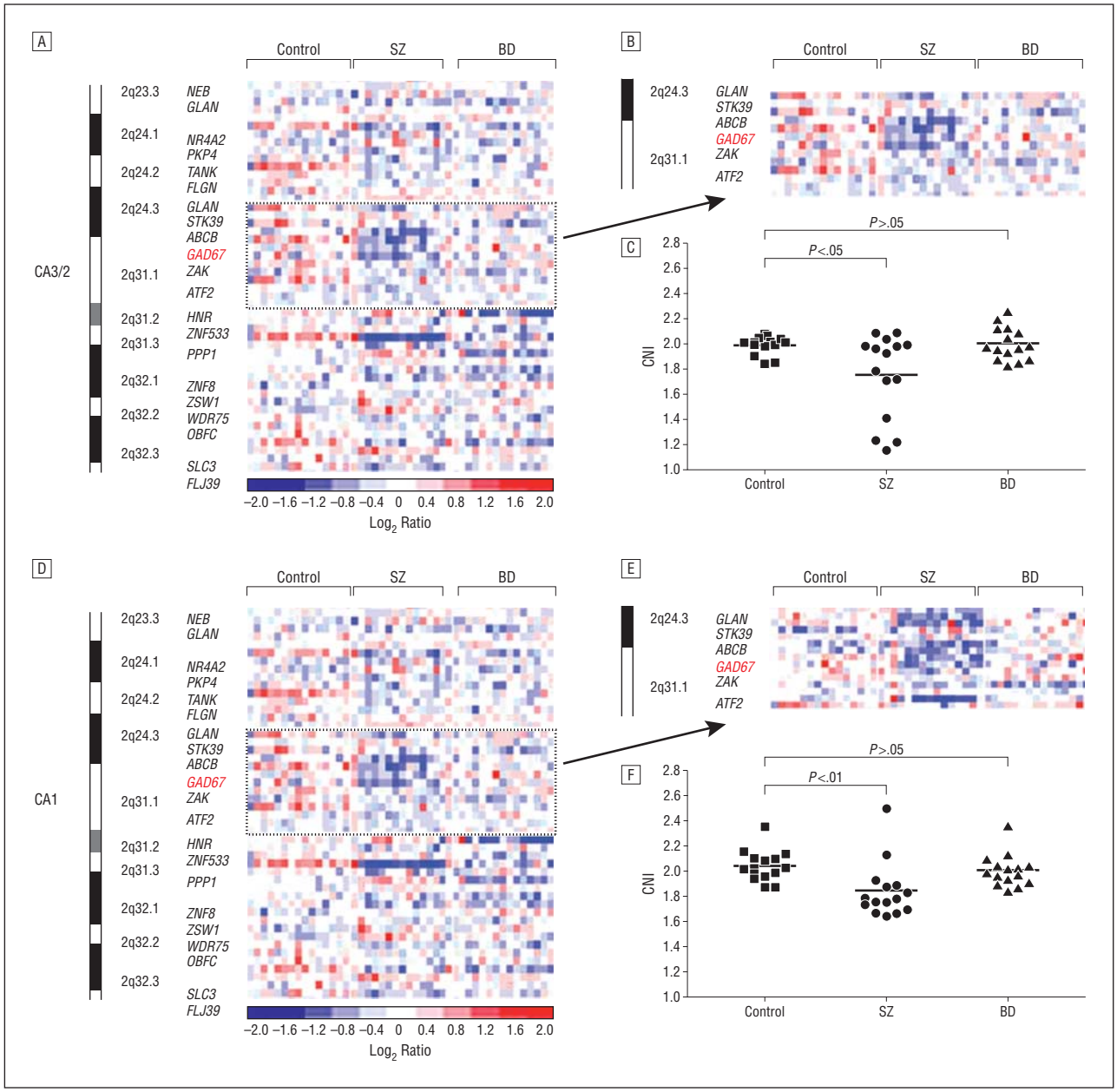

Figure 1. Analysis of DNA copy number changes at the *GAD67*-encompassing segment of chromosome 2q in sectors CA3/2 and CA1 in 15 healthy controls (Control), 15 patients with bipolar disorder (BD), and patients with schizophrenia (SZ). A, Heat map of copy number intensity (CNI) changes in the stratum oriens of sector CA3/2. Each column represents 1 sample and each row represents a specific gene, according to their physical order in the chromosome. Red indicates copy gain, and blue indicates copy loss. Copy numbers are displayed in the $\log_2$ ratio in a color code from –2 to 2. The *GAD67*-encompassing segment is highlighted by the dotted line. The upper arrow points to the close-up view of CNIs at the *GAD67*- encompassing segment at chromosome 2q (B). C, The CNIs of each patient are represented as squares (Control), circles (SZ), and triangles (BD), respectively. The horizontal bars indicate the mean. Mean values were compared using 1-way analysis of variance with the Tukey post hoc test. The SZ group had less copy numbers than the control group (mean [SD], 1.74 [0.27] vs 1.99 [0.05]; $P<.01$) but not the BD group. D, Heat map of CNIs in the stratum oriens of sector CA1. The lower arrow points to the close-up view of CNIs at the *GAD67*-encompassing segment at chromosome 2q (E). F, The CNIs of each patient are represented as squares (Control), circles (SZ), and triangles (BD), respectively. The horizontal bars indicate the mean. Similar to the findings in sector CA3/2, the SZ group in sector CA1 had less copy numbers than the control group (mean [SD], 1.84 [0.15] vs 2.04 [0.09]; $P<.05$) but not the BD group.

gene (*RNaseP*) and for the target gene, 250nM each of the VIC dye (reference)–labeled and FAM dye (target)–labeled gene-specific probe in 1× TaqMan Gene Expression Master Mix. Individual samples were run in triplicate. Real-time data were collected by the OPTICON software. Quantitative real-time PCR data were analyzed using the relative quantification or ΔΔCt method[27] based on DNA copy number ratio of a target gene vs reference gene in a given patient sample relative to matched healthy control sample. Relative quantity (RQ) is $2^{-\Delta\Delta Ct}$, and the copy number is 2 × RQ.

STATISTICAL ANALYSES

GraphPad Prism version 4.0 software was used for statistical analysis. The Fisher exact test and the 1-way analysis of variance with the Tukey post hoc test were used to evaluate differences in copy number for each target gene in the healthy control, schizophrenia, and bipolar disorder groups. Copy number intensities were also correlated with microarray-based gene expression data obtained in an earlier study[16] in which an identical subset of cases was included.

Table 1. Overview of DNA Copy Number Changes of *GAD67* Regulatory Genes in the Stratum Oriens of Sector CA3/2 in Patients With Schizophrenia or Bipolar Disorder

Gene[a]	Cytogenetic Location	Start[b]	End[b]	Size,[c] bp	Mean (SD) CNI		SZ vs Control, %	Mean (SD) CNI for BD	BD vs Control, %	*P* Value[d]	
					Control	SZ				SZ	BD
GAD67 regulation											
GAD67	2q31	171362909	171670922	308013	2.16 (0.18)	1.68 (0.24)	–22	1.61 (0.27)	–25	<.001	<.0001
GAD2	10p11.23	26552050	26618469	66419	2.34 (0.31)	1.76 (0.18)	–24	1.99 (0.56)	–15	.001	>.05
GRIK1	21q22.11	29831916	30234289	402373	1.99 (0.08)	1.90 (0.22)	–5	1.84 (0.16)	–8	.04	.002
GRIK2	6q16.3-q21	101957419	102622143	664724	2.03 (0.09)	2.26 (0.32)	11	1.77 (0.32)	–13	.04	.04
GRIK3	1p34	37006017	37299068	293051	1.94 (0.10)	2.33 (0.26)	20	2.15 (0.11)	11	.04	>.05
DAXX	6p21.3	33362643	33432505	69862	2.15 (0.40)	2.53 (0.50)	17	1.86 (0.22)	–14	.004	>.05
RUNX2	6p21	45427854	45614100	186246	2.01 (0.22)	1.84 (0.11)	–9	1.70 (0.19)	–15	>.05	.003
LEF1	4q23-q25	109114461	109203182	88721	2.05 (0.10)	2.01 (0.17)	–1	1.83 (0.27)	–11	>.05	.002
PAX5	9p13	36843677	36871493	27816	2.16 (0.26)	1.76 (0.27)	–19	1.85 (0.21)	–14	.006	.02
CCND2	12P13	4259419	4276065	16646	2.02 (0.19)	2.05 (0.19)	2	1.72 (0.22)	–15	>.05	.001
HDAC11	3p25.1	13498010	13519073	21063	2.06 (0.31)	2.70 (0.55)	31	2.19 (0.33)	6	<.001	.003
TGFB2	1q41	216594710	216680962	86252	2.06 (0.25)	1.93 (0.17)	–3	1.99 (0.14)	–6	>.05	>.05
TGFBR1	9q22	100911984	100954694	42710	1.99 (0.45)	2.19 (0.45)	10	2.33 (0.21)	17	>.05	>.05
SMAD4	18q21.1	46816802	46848896	32094	1.88 (0.18)	1.61 (0.26)	–14	2.00 (0.33)	7	.05	>.05
Neurogenesis											
FGF3	11q13	69336099	69338136	2037	2.05 (0.46)	1.80 (0.54)	–12	1.50 (0.50)	–27	>.05	.01
FGF9	13q11-q12	21161875	21174668	12793	2.25 (0.35)	2.22 (0.38)	–2	1.79 (0.20)	–20	>.05	<.001
VEGFA	6p12	43850604	43854388	3784	2.25 (0.59)	2.63 (0.64)	17	1.84 (0.43)	–18	.02	.04
NRG1	8p21	31618950	32715614	1096664	2.02 (0.09)	1.72 (0.19)	–15	1.84 (0.16)	–9	<.0001	.02
Cell cycle regulation											
E2F3	6p22	20509888	20598369	88481	2.01 (0.17)	1.78 (0.20)	–11	1.93 (0.17)	–5	.04	>.05
BRCA1	17q21	38450800	38523755	72955	1.90 (0.19)	2.02 (0.30)	–13	1.75 (0.22)	6	>.05	>.05
MBD4	3q21	130631711	130642529	10818	1.99 (0.46)	2.50 (0.48)	25	2.33 (0.53)	17	.002	.02
TP53	17p13.1	7518132	7532486	14354	2.00 (0.38)	1.75 (0.29)	–13	1.53 (0.17)	–24	>.05	.03
ANAPC5	12q24.31	120242043	120270178	28135	1.91 (0.29)	1.94 (0.38)	–1	2.48 (0.51)	29	>.05	.006
DNA damage response											
POLD1	19q13.3	55584616	55605773	21157	2.03 (0.30)	1.60 (0.31)	–22	2.16 (0.21)	8	.02	>.05
POLG2	17q	59909735	59923805	14070	2.33 (0.37)	1.81 (0.22)	–22	1.75 (0.30)	–25	>.05	.02
POLI	18q21.1	50049273	50068773	19500	2.00 (0.22)	1.50 (0.41)	–25	2.30 (0.51)	15	.002	>.05
POLL	10q23	103330169	103412748	82579	2.02 (0.30)	2.11 (0.21)	5	2.26 (0.54)	12	>.05	.04
RPA3	7p22	7642868	7725084	82216	2.07 (0.17)	2.05 (0.39)	–1	2.31 (0.59)	12	>.05	.006

Abbreviations: BD, patients with bipolar disorder; CNI, copy number intensity; Control, healthy controls; SNP, single-nucleotide polymorphism; SZ, patients with schizophrenia.

[a] Gene names derived from National Center for Biotechnology Information UniGene database.

[b] The SNP site is based on the Affymetrix Genome-Wide Human SNP Array 6.0 reference genome.

[c] Length of DNA copy number changes in base pairs (bp).

[d] Determined by use of the Fisher exact test. $P > .05$ is statistically nonsignificant.

RESULTS

COPY NUMBER INTENSITIES IN PATIENTS WITH SCHIZOPHRENIA OR BIPOLAR DISORDER

Genomic copy numbers were determined by calculating the median signal intensities of the healthy controls, the patients with schizophrenia, and the patients with bipolar disorder with respect to normal reference DNAs. As shown in **Figure 1**, the *GAD67*-containing segment at the 2q24.3-2q31.1 chromosomal locus spanned approximately 14.2 megabases. The mean copy number intensity at the *GAD67*-containing locus was 28% lower in the patients with schizophrenia than in the healthy controls. Similarly, lower numbers (15%) also occurred in the CA1 sector of the patients with schizophrenia at the same locus. Patients with bipolar disorder did not show differences in copy number intensities at the 2q24.3-2q31.1 locus in either sector (Figure 1).

COPY NUMBER INTENSITIES AND SPECIFIC TARGET GENES

The analysis of copy number intensities for 28 different target genes associated with the *GAD67* regulatory network (n=14), neurogenesis (n=4), cell cycle regulation (n=5), and the DNA damage response (n=5) for sectors CA3/2 and CA1 are shown in **Table 1** and **Table 2**, respectively. The overall sequence and its specific chromosomal locus are indicated for each gene in Tables 1 and 2. The genes that were chosen as targets for our study satisfied 2 criteria: (1) they must have shown significant differences in gene expression (fold changes),[17,21,24] and (2) the genes must be associated directly or indirectly with the *GAD67* regulatory network that was derived from a network association analysis.[21] Some of the genes that are associated with this network (such as *DLX1* and *DLX2*)[28] and that play an important role in the development and function of GABA cells were not included because they did not show expression changes, and fell below the level of detection because of their very low abundance in the stratum oriens of sectors CA3/2 and CA1 of the human hippocampus.

Table 2. Overview of DNA Copy Number Changes of *GAD67* Regulatory Genes in the Stratum Oriens of Sector CA1 in Patients With Schizophrenia or Bipolar Disorder

Gene[a]	Cytogenetic Location	Start[b]	End[b]	Size,[c] bp	Mean (SD) CNI		SZ vs Control, %	Mean (SD) CNI for BD	BD vs Control, %	*P* Value[d]	
					Control	SZ				SZ	BD
GAD67 regulation											
GAD67	2q31	171362909	171670922	308013	1.95 (0.37)	1.42 (0.51)	–27	1.80 (0.43)	–7	.004	>.05
GAD2	10p11.23	26552050	26618469	66419	2.19 (0.55)	2.20 (0.21)	0	1.83 (0.41)	–16	>.05	>.05
GRIK1	21q22.11	29831916	30234289	402373	2.15 (0.47)	1.90 (0.21)	–11	1.97 (0.11)	–8	>.05	>.05
GRIK2	6q16.3-q21	101957419	102622143	664724	1.99 (0.18)	1.77 (0.31)	–11	1.95 (0.17)	–2	.02	>.05
GRIK3	1p34	37006017	37299068	293051	2.07 (0.12)	2.18 (0.38)	5	1.96 (0.08)	–5	>.05	>.05
DAXX	6p21.3	33362643	33432505	69862	1.91 (0.49)	1.29 (0.36)	–35	1.92 (0.34)	–4	.004	>.05
RUNX2	6p21	45427854	45614100	186246	2.07 (0.23)	1.87 (0.27)	–10	1.88 (0.14)	–9	>.05	>.05
LEF1	4q23-q25	109114461	109203182	88721	2.22 (0.22)	1.98 (0.13)	–11	2.15 (0.23)	–3	.03	>.05
PAX5	9p13	36843677	36871493	27816	2.00 (0.39)	2.28 (0.42)	14	2.26 (0.33)	13	>.05	>.05
CCND2	12P13	4259419	4276065	16646	1.95 (0.32)	2.17 (0.32)	11	1.86 (0.17)	–5	>.05	>.05
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TGFB2	1q41	216594710	216680962	86252	2.07 (0.21)	1.92 (0.29)	–3	2.01 (0.19)	–8	>.05	>.05
TGFBR1	9q22	100911984	100954694	42710	2.12 (0.33)	1.76 (0.31)	–17	2.22 (0.25)	5	.02	>.05
SMAD4	18q21.1	46816802	46848896	32094	2.00 (0.39)	1.53 (0.76)	–24	2.05 (0.46)	0	.02	>.05
Neurogenesis											
FGF3	11q13	69336099	69338136	2037	1.85 (0.33)	2.10 (0.47)	14	2.53 (0.55)	37	>.05	.01
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VEGFA	6p12	43850604	43854388	3784	2.11 (0.47)	2.19 (0.27)	0	2.24 (0.36)	0	>.05	>.05
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MBD4	3q21	130631711	130642529	10818	2.00 (0.24)	2.25 (0.48)	12	2.25 (0.33)	12	>.05	.04
TP53	17p13.1	7518132	7532486	14354	2.16 (0.59)	2.60 (0.74)	20	1.91 (0.39)	–12	>.05	>.05
ANAPC5	12q24.31	120242043	120270178	28135	1.89 (0.32)	2.48 (0.43)	31	2.08 (0.32)	9	.002	>.05
DNA damage response											
POLD1	19q13.3	55584616	55605773	21157	2.11 (0.43)	2.48 (0.67)	17	2.00 (0.35)	–5	>.05	>.05
POLG2	17q	59909735	59923805	14070	2.04 (0.38)	1.82 (0.30)	–11	1.93 (0.30)	–5	>.05	>.05
POLI	18q21.1	50049273	50068773	19500	1.92 (0.30)	1.89 (0.34)	–1	1.93 (0.24)	0	>.05	>.05
POLL	10q23	103330169	103412748	82579	1.89 (0.28)	1.96 (0.31)	4	1.96 (0.32)	4	>.05	>.05
RPA3	7p22	7642868	7725084	82216	1.99 (0.15)	2.03 (0.24)	2	2.07 (0.18)	4	>.05	>.05

Abbreviations: BD, patients with bipolar disorder; CNI, copy number intensity; Control, healthy controls; SNP, single-nucleotide polymorphism; SZ, patients with schizophrenia.

[a] Gene names derived from National Center for Biotechnology Information UniGene database.

[b] The SNP site is based on the Affymetrix Genome-Wide Human SNP Array 6.0 reference genome.

[c] Length of DNA copy number changes in base pairs (bp).

[d] Determined by use of the Fisher exact test. $P > .05$ is statistically nonsignificant.

GAD67 Regulation

Significant changes in copy number intensity in *GAD67* regulatory genes were observed in 9 of 14 patients with schizophrenia (64%) and in 7 of 14 patients with bipolar disorder (50%) (Tables 1 and 2). The mean CNI for this gene was also decreased by 22% and 25%, respectively, in patients with schizophrenia and patients with bipolar disorder. In sector CA1, however, 50% of patients with schizophrenia showed significant CNI changes in *GAD67* regulatory genes, whereas the patients with bipolar disorder showed no changes (Tables 1 and 2).

Heat maps of copy number intensities for specific chromosomal loci were plotted for several genes in the *GAD67* regulatory network (Figure 1). As suggested by our earlier gene expression profile (GEP) study,[21] *GAD67* expression was significantly decreased in sector CA3/2 of patients with schizophrenia and patients with bipolar disorder (Table 1). As shown in **Figure 2**A, the SNP arrays for *GAD67*, *HDAC11*, *DAXX*, *PAX5*, *RUNX2*, and *CCND2* showed changes in copy number intensities in patients with schizophrenia and patients with bipolar disorder that are remarkably similar to those previously reported for a GEP study[21] at this same locus of CA3/2. For example, *GAD67* expression is reduced in both patients with schizophrenia and patients with bipolar disorder, *HDAC11* and *DAXX* expression increased only in patients with schizophrenia, and both *PAX5* and *RUNX2* expression significantly decreased in patients with bipolar disorder. Two exceptions are *PAX5*, for which expression was only significantly lower in patients with bipolar disorder, and *CCND2*, for which transcripts were increased in patients with schizophrenia at this locus (Figure 2A). In the CA1 sector, copy number intensities for *GAD67* are significantly decreased only for patients with schizophrenia, not for patients with bipolar disorder (Figure 2B), but this pattern is virtually identical to that seen for the GEP findings previously reported for the same cases.[16] Copy number intensities for *HDAC11* increased by 78% (Table 2), but the copy number intensities for the other genes do not show significant changes.

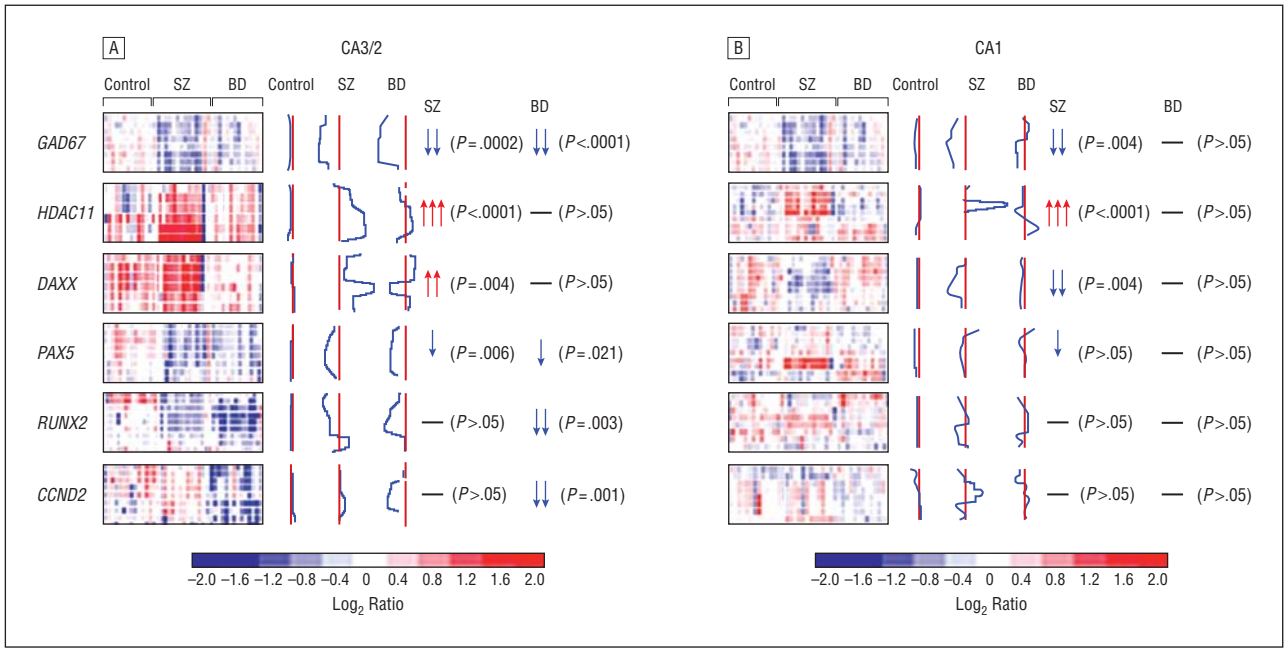

Figure 2. Heat Maps of DNA copy number changes for *GAD67* regulatory genes in the stratum oriens of sector CA3/2 (A) and sector CA1 (B). The copy number intensities of 6 critical *GAD67* regulatory genes are compared by performing the Fisher exact test between 15 healthy controls (Controls), 15 patients with schizophrenia (SZ), and 15 patients with bipolar disorder (BD). In the heat maps, red represents copy gain, and blue represents copy loss, in units of $\log_2$. The blue curves in the middle graphs indicate the degree of gain (right) or loss (left) of each gene. The red vertical line indicates a copy number of 2. Also shown are the probabilities that changes are significant; the arrows indicate whether copy number intensities are increased (red arrows) or decreased (blue arrows).

Copy number intensities for *GAD65* (*GAD2*), *GRIK1*, *RUNX2*, *PAX5*, and *SMAD4* were also decreased in the CA3/2 sector of patients with schizophrenia, whereas *GRIK2*, *GRIK3*, *HDAC11*, and *DAXX* all had higher copy number intensities. As shown in **Table 3**, the copy number intensity changes for these target genes in the CA3/2 sector of patients with schizophrenia were similar in direction to those reported with a GEP.[16,21] In the CA3/2 sector of patients with bipolar disorder (Table 1), the copy numbers of *GRIK1*, *GRIK2*, *GAD65*, *LEF1*, *DAXX*, *RUNX2*, and *PAX5* were also decreased, and most showed a direction of change consistent with the earlier GEP data for these same genes[21] (Table 3).

Neurogenesis

The 4 genes in the neurogenesis cluster were chosen because they had shown significant changes in their GEPs, and our modeling suggested that they are probably important to the regulation of the cell cycle.[19] *FGF3*,[29] *FGF9*,[30,31] *VEGF*,[32] and *NRG1*[33] are key players in the growth and differentiation of a wide variety of cells and tissues, including the hippocampus,[34] and are believed to increase the risk for schizophrenia.[35,36] In CA3/2 sector of patients with bipolar disorder, these genes showed significant decreases in copy number intensity, and these changes occurred in the same direction as those observed when a GEP was undertaken (Table 3). In the patients with schizophrenia, *VEGF* showed increases of copy number intensity in the CA3/2 sector, and this direction of change is similar to that seen in the GEPs for the same genes. *NRG1* showed significant reductions in copy number intensity; however, the transcripts for this gene were significantly changed in the opposite direction for the GEP data (Table 3).

Cell Cycle and DNA Damage Response

Copy number intensities for cell cycle regulation and the DNA damage response showed significant changes in the CA3/2 sector of patients with schizophrenia and patients with bipolar disorder (Table 1). The copy number intensity of *E2F3* in patients with schizophrenia was significantly reduced, and this change was in the same direction as that observed for transcripts in the GEP study.[21] The patients with bipolar disorder showed only 1 gene (*MBD4*) with significant changes in copy number intensity and gene expression, both of which were increased; however, this similar change was seen for this gene in sectors CA3/2 and CA1 of both groups of patients. Overall, the changes observed for copy number intensities in the cell cycle and DNA damage response categories showed much less consistency when compared with the GEP data for these same genes.

To validate the copy number intensity changes identified by SNP array, we performed quantitative PCR analysis for the candidate gene *GAD67* using gDNA from microdissected tissues (obtained from 6 patients with schizophrenia, 6 patients with bipolar disorder, and 6 healthy controls). In sector CA3/2, the copy number for the *GAD67* gene also decreased by 34% and 37%, respectively, in patients with schizophrenia and patients with bipolar disorder, which is in complete agreement with the SNP data. In sector CA1, the copy number for the *GAD67* gene decreased by 59% in patients with schizophrenia, although there was only a 10% decrease in patients with bipolar disorder (eFigure 2). These results have demonstrated the overall validity of the copy number status determined by SNP microarrays, and they also establish that *GAD67* is the gene with one of the most fre-

Table 3. Copy Number Changes and Corresponding Gene Expression Fold Changes in the Stratum Oriens of Sectors CA3/2 and CA1

	CA3/2				CA1			
	Schizophrenia		Bipolar Disorder		Schizophrenia		Bipolar Disorder	
Gene[a]	CNI Changes, %	Fold Changes	CNI Changes, %	Fold Changes	CNI Changes, %	Fold Changes	CNI Changes, %	Fold Changes
GAD67 regulation								
GAD67	–22	–2.81	–25	–9.59	–27	–3.27	–7	0.00
GAD65	–24	–2.29	–15	–3.00	0	–1.11	–16	0.00
GRIK1	–5	–1.39	–8	–1.36	–11	–1.35	–8	–1.47
GRIK2	11	1.50	–13	–1.20	–11	0.00	–2	0.00
GRIK3	20	1.60	11	0.00	5	0.00	–5	0.00
DAXX	17	1.30	–14	0.00	–35	0.00	–4	0.00
RUNX2	–9	0.00	–15	–2.80	–10	0.00	–9	0.00
LEF1	–1	0.00	–11	–2.10	–11	–2.08	–3	–2.23
PAX5	–19	0.00	–14	–1.48	–19	0.00	–13	0.00
CCND2	2	1.44	–15	–2.18	11	0.00	–5	0.00
HDAC11	31	1.49	6	0.00	78	0.00	5	0.00
TGFB2	–3	1.43	–6	0.00	–3	0.00	–8	3.00
TGFBR1	10	1.30	17	0.00	–17	0.00	5	0.00
SMAD4	–14	0.00	7	2.30	22	–1.52	5	–1.34
Neurogenesis								
FGF3	–12	0.00	–27	–1.62	–24	0.00	0	0.00
FGF9	–2	0.00	–20	–11.33	14	0.00	37	0.00
VEGFA	17	2.13	–18	–1.41	0	0.00	0	–1.47
NRG1	–15	1.59	–19	–1.77	–14	0.00	–13	0.00
Cell cycle regulation								
E2F3	–11	–1.77	–5	0.00	4	0.00	0	2.02
BRCA1	–13	0.00	6	0.00	–15	–1.92	0	0.00
MBD4	25	2.91	17	1.75	12	1.77	12	1.97
TP53	–13	0.00	–24	–1.51	20	0.00	–12	0.00
ANAPC5	–1	0.00	29	3.13	31	0.00	9	4.37
DNA damage response								
POLD1	–22	–2.87	8	0.00	17	0.00	–5	0.00
POLG2	–2	0.00	–25	–1.48	–11	0.00	–5	0.00
POLI	–25	1.84	15	0.00	–1	0.00	0	0.00
POLL	5	0.00	12	–1.30	4	0.00	4	0.00
RPA3	–1	0.00	12	1.35	2	1.40	4	0.00

[a] Target genes (n = 28) are divided into 4 clusters groups: the *GAD67* regulatory network, neurogenesis, the cell cycle regulation, and the DNA damage response. Each column indicates copy number intensity (CNI) changes and corresponding gene expression fold changes.

quent genetic alterations in schizophrenia and bipolar disorder.

ASSOCIATION ANALYSIS BETWEEN COPY NUMBER INTENSITIES AND GENE EXPRESSION

Do DNA copy number intensities reflect gene expression patterns that we have identified in patients with schizophrenia and patients with bipolar disorder within the loci of interest? In sector CA3/2 of both the schizophrenia and bipolar disorder groups, the majority of target genes showed abnormally high or low levels of mRNA expression, whereas in sector CA1, both groups showed a paucity of significant expression changes (Table 3). When decreases or increases of copy numbers are observed, the changes seen could be related to either deletions or duplications, respectively, of chromatin material. The genes showing significant correlations between copy number intensities and gene expression for the *GAD67* regulatory genes (**Figure 3**) were generally quite different for the schizophrenia and bipolar disorder groups. As shown in Table 3, the patients with bipolar disorder showed no expression changes for *GRIK3*, *DAXX*, *HDAC11*, *TGFB2*, and *TGFBR1,* whereas all of the other genes showed significant expression changes. In patients with schizophrenia, *RUNX2*, *LEF1*, *PAX5*, and *SMAD4* did not show expression changes. Overall, there was a diagnostic selectivity for the target genes associated with decreased *GAD67* expression in schizophrenia vs bipolar disorder.[21]

When the GEP data and the copy number intensity data (Table 3) were analyzed using linear regression analyses (Figure 3), a robust correlation was observed when both groups of patients were combined ($r=0.692$; $P=.0001$) or when the patients with schizophrenia ($r=0.649$; $P=.0003$) and the patients with bipolar disorder ($r=0.772$; $P=.0002$) were separately compared with the healthy controls (Figure 3). For a majority of the target genes, the changes in copy number intensities and the fold changes tended to occur in the same direction, although the magnitudes of the respective changes were, in some instances, dramatically different. For example, mRNA expression for *FGF9* was decreased 11-fold in patients with bipolar disorder, whereas the loss of copy number intensity for this gene was only 20%. Disparities of this type contributed to the variance in the data and resulted in a reduction in the *r* correlation coefficient, even when the correlations were statistically significant.

As shown in **Table 4** and **Figure 4**, when the target genes were separated according to those directly related to the *GAD67* regulatory network and those not related to it (ie, genes associated with growth factors, cell cycle, and DNA repair), much higher correlations between copy number intensity changes and mRNA levels were ob-

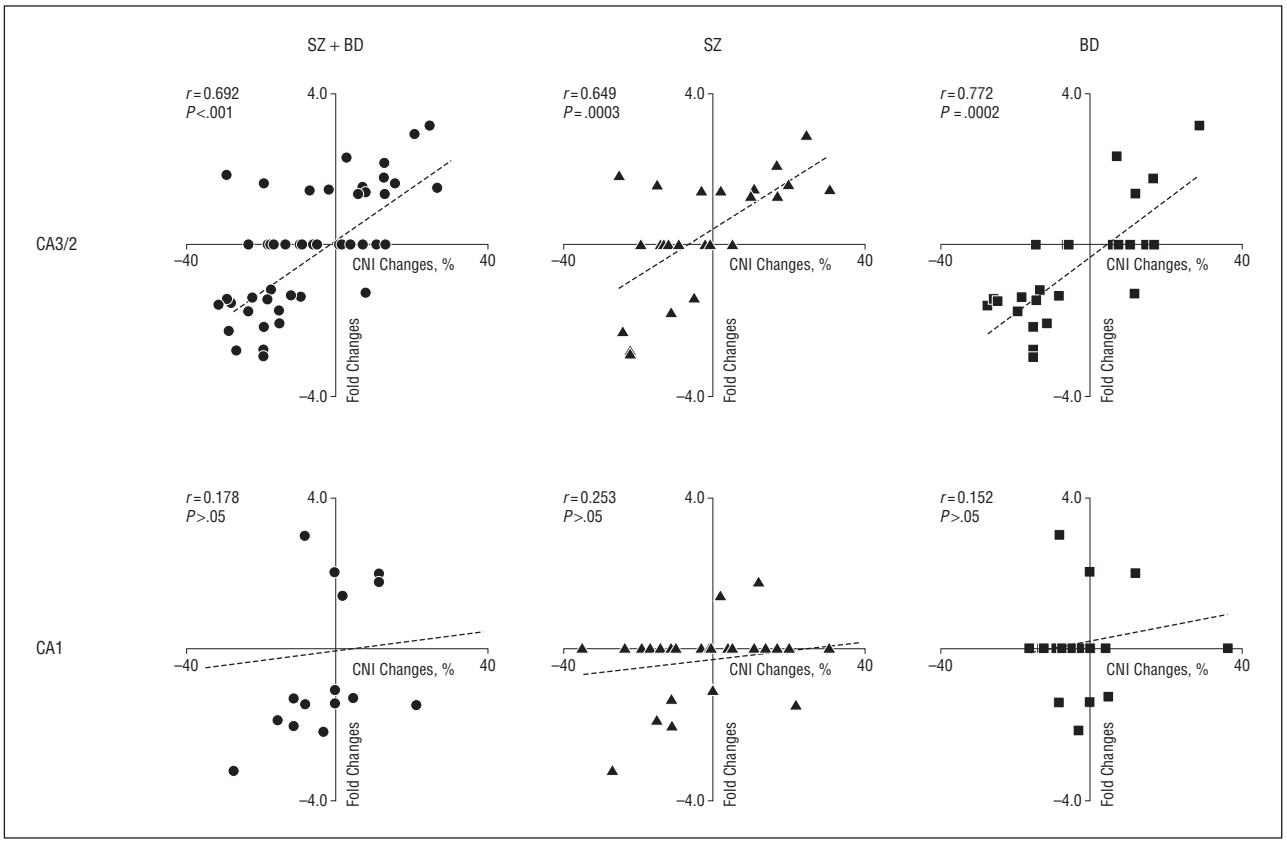

Figure 3. Association of fold changes as a measure of gene expression and DNA copy number intensity (CNI) changes in the stratum oriens of sectors CA3/2 and CA1.The x-axis shows CNI changes, and the y-axis displays fold changes, from microarray data. Individual data points are connected with a line using linear regression analysis performed with GraphPad Prism version 4.0. In the stratum oriens of sector CA3/2, significant associations between CNI changes and fold changes are evident in patients with schizophrenia (SZ), patients with bipolar disorder (BD), and combined patients (SZ+BD), respectively. There are no significant associations between CNI changes and fold changes in any patient groups in sector CA1.

served in the former ($r=0.712$; $P=.0001$) than in the latter ($r=0.492$; $P=.008$) of sector CA3/2 but not sector CA1 ($r=0.067$ and $r=0.225$, respectively). These data suggest that the expression of genes in the *GAD67* network in the CA3/2 sector is tightly linked to the status of genomic integrity at this locus.

The linear regression analyses of target genes in sector CA1 did not reveal any correlation between gene expression and copy number intensity changes. There are many genes showing notable expression changes but no copy number intensity changes, suggesting that a dissociation between copy number intensities and gene expression changes may occur in sector CA1. As with sector CA3/2, however, a subset of genes in sector CA1 not related to *GAD67* regulation did not show correlations with copy number intensities (Figure 4). These results demonstrate a striking subregional difference in the association of copy number intensities with gene expression changes.

COMMENT

There is increasing evidence supporting the involvement of copy number variants in the etiology of schizophrenia,[9,37-39] although it is not clear what mechanisms may be related to these changes. Most of these reports have employed genome-wide screening for copy number variants, and only a few have concentrated on specific target genes.[40] Although there have been preliminary attempts at linking copy number variants with expression data for specific genes in cancer research,[41,42] to date, no study of the brain has attempted to associate the occurrence of copy number intensities for specific target genes with the expression of their respective mRNAs. The results described herein suggest that copy number intensity changes for specific target genes and their associated mRNAs vary not only in a diagnosis-specific way but also in a circuitry-based manner.

GAD67 plays an important role in the activity of GABA neurons and their dysfunction in schizophrenia. Although one association study[37] concluded that there was a link between the *GAD67* gene and childhood-onset schizophrenia, another study,[43] using a Danish cohort, provides evidence that, in this sample, there is no link to schizophrenia but, possibly, a link to bipolar disorder. Another gene, neuregulin 1 (*NRG1*), may be functionally involved in the regulation of *GAD67* activity.[44] Interestingly, *NRG1* also showed significant changes in both copy number intensity and gene expression, in patients with schizophrenia and patients with bipolar disorder. This gene appears to have a weak association with schizophrenia when haplotype-based *P* values were included in the analyses, and there was no evidence of between-study heterogeneity.[45] These studies underscore

Table 4. Copy Number Changes and Corresponding Gene Expression Fold Changes for *GAD67* and Non-*GAD67* Regulatory Genes in the Stratum Oriens of Sectors CA3/2 and CA1

	CA3/2				CA1			
	Schizophrenia		Bipolar Disorder		Schizophrenia		Bipolar Disorder	
Gene[a]	CNI Changes, %	Fold Changes	CNI Changes, %	Fold Changes	CNI Changes, %	Fold Changes	CNI Changes, %	Fold Changes
GAD67 regulation								
GAD67	–22	–2.81	–25	–9.59	–27	–3.27	–7	0.00
GAD65	–24	–2.29	–15	–3.00	0	–1.11	–16	0.00
GRIK1	–5	–1.39	–8	–1.36	–11	–1.35	–8	–1.47
GRIK2	11	1.50	–13	–1.20	–11	0.00	–2	0.00
GRIK3	20	1.60	11	0.00	5	0.00	–5	0.00
DAXX	17	1.30	–14	0.00	–35	0.00	–4	0.00
RUNX2	–9	0.00	–15	–2.80	–10	0.00	–9	0.00
LEF1	–1	0.00	–11	–2.10	–11	–2.08	–3	–2.23
PAX5	–19	0.00	–14	–1.48	–19	0.00	–13	0.00
CCND2	2	1.44	–15	–2.18	11	0.00	–5	0.00
HDAC11	31	1.49	6	0.00	78	0.00	5	0.00
TGFB2	–3	1.43	–6	0.00	–3	0.00	–8	3.00
TGFBR1	10	1.30	17	0.00	–17	0.00	5	0.00
SMAD4	–14	0.00	7	2.30	22	–1.52	5	–1.34
Non-*GAD67* regulation								
FGF3	–12	0.00	–27	–1.62	–24	0.00	0	0.00
FGF9	–2	0.00	–20	–11.33	14	0.00	37	0.00
VEGFA	17	2.13	–18	–1.41	0	0.00	0	–1.47
NRG1	–15	1.59	–19	–1.77	–14	0.00	–13	0.00
E2F3	–11	–1.77	–5	0.00	4	0.00	0	2.02
BRCA1	–13	0.00	6	0.00	–15	–1.92	0	0.00
MBD4	25	2.91	17	1.75	12	1.77	12	1.97
TP53	–13	0.00	–24	–1.51	20	0.00	–12	0.00
ANAPC5	–1	0.00	29	3.13	31	0.00	9	4.37
POLD1	–22	–2.87	8	0.00	17	0.00	–5	0.00
POLG2	–2	0.00	–25	–1.48	–11	0.00	–5	0.00
POLI	–25	1.84	15	0.00	–1	0.00	0	0.00
POLL	5	0.00	12	–1.30	4	0.00	4	0.00
RPA3	–1	0.00	12	1.35	2	1.40	4	0.00

[a] Target genes (n = 28) are divided into 2 clusters groups: the *GAD67* regulatory network and the non-*GAD67* regulatory network. Each column indicates copy number intensity (CNI) changes and corresponding gene expression fold changes.

the difficulties inherently present when attempting to relate copy number intensities derived from blood samples in population studies to copy number intensities obtained from brain tissue in studies of the molecular regulation of neural circuitry. Generally speaking, the genes included in the present study cannot be thought of as genetically transmitted risk factors, even when both copy number intensities and mRNA expression show robust changes, because the study design is fundamentally different from that used in association studies. The information gathered in our study is most useful in identifying the ways in which the molecular regulation of complex circuits may be abnormal, particularly when combined with parallel rodent modeling.[46]

The target genes examined in our study are involved in the functional maintenance of hippocampal GABA cells that show abnormal expression of *GAD67* in schizophrenia and bipolar disorder.[17,21] They include networks that regulate *GAD67* expression, neurogenesis, the cell cycle, and the DNA damage response. In the present study, most of these genes showed significant increases or decreases of copy number intensities at their respective chromosomal loci. The largely robust nature of these copy number intensities and their correlation with the respective expression changes suggest that they may be linked to the regulation of functional differentiation and genomic integrity in hippocampal GABA cells. Widespread deletions along the *GAD67*-encompassing segment of chromosome 2q (ie, 2q24.3-2q31.1), where *GAD67* is encoded,[47] have been identified in patients with schizophrenia but not in patients with bipolar disorder. The fact that *GAD67* expression is significantly reduced in sector CA3/2 of both disorders suggests that these broad-based deletions on the 2q chromosome may be only one of the many mechanisms involved in the decrease of *GAD67* expression.

The results from quantitative PCR showed a decreased copy number intensity of *GAD67* in patients with schizophrenia or bipolar disorder, which was consistent with the results from SNP arrays. These findings demonstrate that a decreased copy number intensity of *GAD67* in the hippocampus could be a prevalent genetic change in the majority of patients with schizophrenia or bipolar disorder. More importantly, there was a significant correlation between DNA loss and RNA underexpression of the *GAD67* gene, suggesting that the transcript level of this gene may be regulated by its DNA copy number.

In sector CA3/2, the direction of change for copy number intensities showed a high degree of correspondence with the expression changes for the respective target genes. For example, in patients with schizophrenia, *HDAC11* and *DAXX* both showed increased copy number intensities and expression changes. In patients with bipolar disorder, however, these parameters for *PAX5* and *RUNX2*

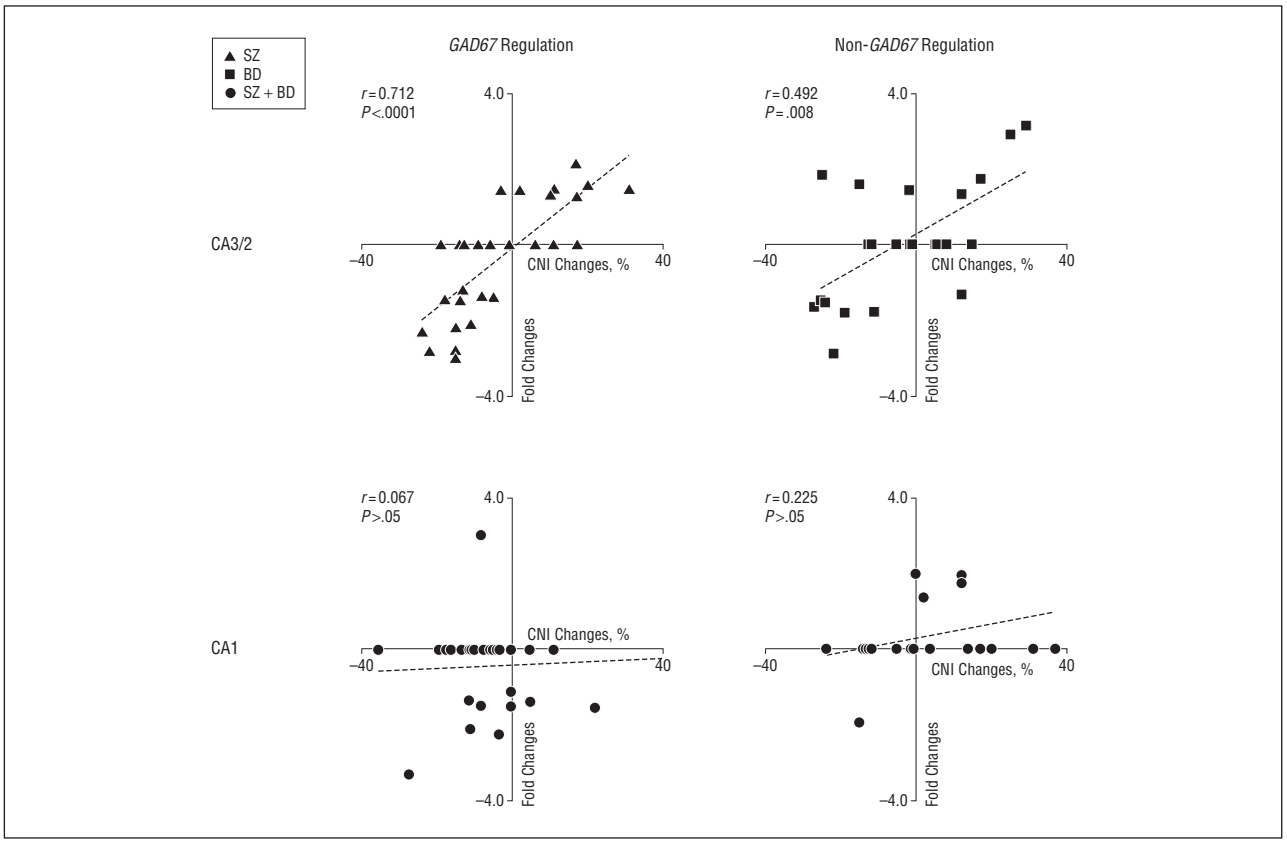

Figure 4. Association of fold changes as a measure of gene expression and DNA copy number intensity (CNI) changes for *GAD67* and non-*GAD67* regulatory genes in the stratum oriens of sectors CA3/2 and CA1. The x-axis shows CNI changes, and the y-axis displays fold changes, from microarray data. Individual data points are connected with a line using linear regression analysis performed with GraphPad Prism version 4.0. In sector CA3/2, both clusters showed a significant association between CNI changes and fold changes, but the *GAD67* regulatory network has a higher correlation efficiency than does the non-*GAD67* regulatory network. However, there is no significant association between CNI changes and fold changes in any gene cluster in sector CA1.

were both decreased. Indeed, the regression analyses provide compelling evidence to support the idea that these 2 variables are interrelated. The relationship between copy number intensities and gene expression changes was most striking for the genes directly involved in *GAD67* regulation compared with the genes more indirectly involved in neurogenesis, cell cycle regulation, and the DNA damage response.

The fact that the nature of the changes in copy number intensity and gene expression for the target genes was quite different for the 2 disorders may reflect the fundamentally different nature of the molecular abnormalities found in the GABA cells of sector CA3/2 for each disorder. It seems unlikely that psychotropic medications are responsible for these differences because the 2 groups showed similar drug exposure histories (eTable). It seems more likely that the unique changes in copy number intensity and gene expression in sector CA3/2 might be related to differences in "risk genes" for the 2 respective disorders.

Unlike the copy number intensities in sector CA3/2, those in sector CA1 of patients with schizophrenia or bipolar disorder did not show any correlation with gene expression changes. Only 20% to 30% of the target genes associated with cell cycle regulation and DNA repair in sector CA1 of patients with schizophrenia or bipolar disorder showed significant changes in expression (Table 3).

This obvious difference between the findings in sector CA3/2 and the findings in sector CA1 is consistent with the idea that the local tissue environment surrounding a cell may play a critical role in influencing its profile of transcriptional activity. In the hippocampus of patients with schizophrenia or bipolar disorder, the concentration of significant findings in the stratum oriens of sector CA3/2, but not sector CA1, can potentially be explained by differences in the molecular activity within neurons that comprise the microcircuitry at these 2 respective sites.[24] For example, the stratum oriens of sector CA3/2, but not sector CA1, receives a rich glutamatergic projection from the basolateral amygdala, and these fibers are believed to play an important role in the integration of emotion and cognition, particularly in relation to stressful events.[48] Other systems that are unique to this locus include GABAergic fibers that originate in the septal nuclei[49] and contribute to the regulation of oscillatory rhythms.[50]

The stratum oriens is unique because the exclusive neuronal cell type in this layer is the GABAergic interneuron. These latter cells receive a robust and specific projection from the basolateral amygdala,[51] which exerts an important influence on membrane properties and the action potential firing rate in fast-spiking inhibitory cells.[46] Based on the present findings, basolateral amygdala projections to the stratum oriens of the CA3/2 locus may contribute to the functional and genomic integrity of GABAergic interneurons at this locus, presumably through synaptic or modulatory mechanisms. The *GAD67* regulatory network includes 3 kainate-sensitive glutamate re-

ceptor (*KAR*) genes (*GRIK1*, *GRIK2*, and *GRIK3*) that encode the GluR5, 6, and 7 subunits, respectively, for this receptor. Abnormal expression and activity of *KAR* in the hippocampus are believed to play an important role in schizophrenia.[52,53] Because the *GAD67* regulatory network is linked to the canonical clusters associated with the cell cycle and the DNA damage response, the synaptically mediated influence of electrical activity generated within the trisynaptic pathway could contribute, at least theoretically, to parallel changes in copy number intensity and gene expression for the target genes associated with the regulation of *GAD67* in GABA cells at this locus. These changes could include either deletions or duplications of genomic sequences in these target genes.

Most of our knowledge regarding the relationship between the sensing and repair of DNA has come from proliferating cell populations, like those active during embryogenesis.[54] Little or nothing is known about the regulation of the cell cycle or the DNA damage response in postmitotic cells in the adult brain. Many believe that terminal differentiation involves an extensive reprogramming of the genome, so that genes that are relevant to interneuron function, like those associated with *GAD67* regulation, are transcribed, while other genes are permanently silenced.[55]

The mechanisms associated with the repair of damaged DNA include pivotal target genes, such as *CCND2*, *E2F3*, *MBD4*, and *HDAC*, that may help to link the differentiation of mature GABA cells to their ability (or inability) to repair damaged DNA and preserve their genomic and functional integrity. To our knowledge, the present study provides the first evidence that deletions and/or duplications are present in terminally differentiated neurons in the adult hippocampus and that such changes may also contribute to the aberrant expression of key genes involved in the normal and abnormal functioning of hippocampal GABA cells.

In summary, the analyses reported herein demonstrate that there is considerable overlap between copy number intensities and mRNA expression for target genes associated with *GAD67* regulation at a key locus within the hippocampi of patients with schizophrenia or bipolar disorder. Elucidating cell type–specific and locus-specific associations of genes comprising the *GAD67* regulatory network in hippocampal GABA cells with those associated with the cell cycle and DNA repair could help to explain the presence of unique cellular endophenotypes in each of these 2 disorders.[56] The genomic integrity and differentiation of tissue-specific functions in postmitotic GABA cells, and their potential relationship with age-related changes in health and disease,[54] are issues that will require further study in many different forms of neuropsychiatric disease.

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Correspondence: Francine M. Benes, MD, PhD, McLean Hospital, 115 Mill St, Belmont, MA 02478 (fbenes@mclean.harvard.edu).
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