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Role of glutamic acid decarboxylase 67 in regulating cortical parvalbumin and GABA membrane transporter 1 expression: Implications for schizophrenia

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Abstract

Markers of GABA neurotransmission are altered in multiple regions of the neocortex in individuals with schizophrenia. Lower levels of glutamic acid decarboxylase 67 (GAD67) mRNA and protein, which is responsible for most cortical GABA synthesis, are accompanied by lower levels of GABA membrane transporter 1 (GAT1) mRNA. These alterations are thought to be most prominent in the parvalbumin (PV)-containing subclass of interneurons, which also contain lower levels of PV mRNA. Since GAT1 and PV each reduce the availability of GABA at postsynaptic receptors, lower levels of GAT1 and PV mRNAs have been hypothesized to represent compensatory responses to an upstream reduction in cortical GABA synthesis in schizophrenia. However, such cause-and-effect hypotheses cannot be directly tested in a human illness. Consequently, we used two mouse models with reduced GAD67 expression specifically in PV neurons (PV^{GAD67+/-}) or in all interneurons (GABA^{GAD67+/-}) and quantified GAD67, GAT1 and PV mRNA levels using methods identical to those employed in studies of schizophrenia. Cortical levels of PV or GAT1 mRNAs were not altered in PV^{GAD67+/-} mice during postnatal development or in adulthood. Furthermore, cellular analyses confirmed the predicted reduction in GAD67 mRNA, but failed to show a deficit in PV mRNA in these animals. Levels of PV and GAT1 mRNAs were also unaltered in GABA^{GAD67+/-} mice. Thus, mouse lines with cortical reductions in GAD67 mRNA that match or exceed those present in schizophrenia, and that differ in the developmental timing and cell typespecificity of the GAD67 deficit, failed to provide proof-of-concept evidence that lower PV and GAT1 expression in schizophrenia are a consequence of lower GAD67 expression. Together, these findings suggest that the correlated decrements in cortical GAD67, PV and GAT1 mRNAs in schizophrenia may be a common consequence of some other upstream factor.

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Conflict of Interest

All other authors report no competing interests.

Keywords

glutamic acid decarboxylase; GABA transporter; parvalbumin; prefrontal cortex; schizophrenia

Introduction

Schizophrenia is associated with alterations in several markers of GABA neurotransmission in multiple cortical regions, including prefrontal, primary motor, primary visual, and anterior cingulate cortices (Akbarian and Huang, 2006; Benes and Berretta, 2001; Fung et al., 2010; Hashimoto et al., 2008). Both mRNA (Akbarian et al., 1995; Duncan et al., 2010; Straub et al., 2007; Volk et al., 2000) and protein (Curley et al., 2011; Guidotti et al., 2000) levels of the 67 kilodalton isoform of glutamic acid decarboxylase (GAD67), the enzyme responsible for the majority of cortical GABA synthesis, are lower in the neocortex of subjects with schizophrenia. Levels of GABA membrane transporter 1 (GAT1) mRNA, which removes GABA from the extracellular space, are also lower in schizophrenia (Hashimoto et al., 2008; Volk et al., 2001; Ohnuma et al., 1999). These deficits in GABA synthesis and reuptake appear to be most prominent in the fast-spiking, parvalbumin- (PV) containing subclass of interneurons that also contain lower levels of PV mRNA (Hashimoto et al., 2003).

Through different mechanisms, PV and GAT1 can each reduce the amount of GABA available at postsynaptic receptors; therefore, lower expression of PV and GAT1 mRNAs in schizophrenia has been hypothesized to be a compensatory response to counteract insufficient GABA synthesis (Lewis et al., 2005). PV is a slow Ca^{2+} buffer that increases the rate at which residual Ca^{2+} transients decay in GABA nerve terminals after neurotransmitter release (Collin et al., 2005; Muller et al., 2007). Therefore, during repetitive firing, reductions in PV levels in schizophrenia would increase the amount of calcium available to facilitate neurotransmitter release, resulting in increased synaptic GABA levels (Lewis et al., 2005). In fact, mice deficient in PV show enhanced GABA release from fast-spiking neurons (Collin et al., 2005; Vreugdenhil et al., 2003).

GAT1, one of four high-affinity Na^+/Cl^- transporters that take up extracellular GABA, is the most abundant membrane GABA transporter in the cortex (Conti et al., 1998). Consistent with a role for GAT1 in removing extracellular GABA from the synaptic space, in the hippocampus of GAT1 KO mice $^3\text{[GABA]}$ uptake is virtually absent and tonic GABA^A receptor-mediated current is substantially increased (Jensen et al., 2003). Blockade of GAT1 also increases the duration of evoked IPSCs at inhibitory synapses (Overstreet and Westbrook, 2003).

Together, these findings suggest that reducing PV and GAT1 expression in schizophrenia could serve as compensatory responses to augment the postsynaptic effects of the lower GABA levels (Lewis et al., 2005). Consistent with this hypothesis, the alterations in both PV and GAT1 mRNAs are significantly correlated with GAD67 mRNA changes in schizophrenia, and all three transcripts exhibit similar laminar patterns of reduction in the cortex (Volk et al., 2001; Hashimoto et al., 2003). However, whether lower GAD67 mRNA is the cause of downstream reductions in PV and GAT1 expression cannot be directly tested in the illness.

Thus, the goal of this study was to use experimental manipulations in mice in order to perform a proof-of-concept test of the hypothesis that lower GAD67 expression in PV neurons leads to lower PV and GAT1 mRNA levels in schizophrenia. Accordingly, we quantified developmental and adult levels of PV and GAT1 mRNAs in a mouse model with reduced GAD67 expression specifically in PV neurons, as well as in a model with lower

GAD67 mRNA in all GABA neurons. These two mouse lines provide a robust test of the human disease hypothesis since they differ in the magnitude, developmental timing and cell type-specificity of reduced GAD67 mRNA expression. In order to maximize the utility of the mouse models for testing a hypothesis in a human disease, we made in the mice the same measures using the same techniques that had been previously used in studies of schizophrenia, an approach previously shown to be successful in other proof-of-concept tests of disease mechanism hypotheses in schizophrenia (Eggan et al., 2012; Hashimoto et al., 2005).

Materials and Methods

Animals

PV-specific GAD67 heterozygous (PV^{GAD67+/-}) mice—*Pvalb*^{cre/cre} mice (Hippenmeyer et al., 2005; Cardin et al., 2009), in which cre expression is driven by the endogenous mouse *Pvalb* promoter, were obtained from Silvia Arber (Friedrich Miescher Institute, Basel, Switzerland). *Pvalb*^{cre/cre} mice were bred with *Gad1*^{lox/+} mice obtained from Richard Palmiter (Chattopadhyaya et al., 2007) to produce litters containing mutant mice with one copy of the *Gad1* gene (which codes for GAD67) removed in PV neurons (*Pvalb*^{cre/+}*Gad1*^{lox/+}; subsequently referred to as PV^{GAD67+/-} mice) and control mice (*Pvalb*^{cre/+}*Gad1*^{+/+}; subsequently referred to as PV^{GAD67+/+}). Animals were euthanized at 3 different ages: postnatal day 17 [P17; n=5 (2 males and 3 females) per genotype], P30 [n=6 (4 males and 2 females) per genotype], and P50 [n=6 (3 males and 3 females) per genotype], and brains were removed, frozen, and stored at -80°C. Coronal sections from each brain were cut (12 µm), thaw mounted onto SupraFrost slides (Fisher Scientific, Pittsburgh, PA), and stored at -80°C.

Germ-line GAD67 heterozygous (GABA^{GAD67+/-}) mice—GAD67 heterozygous (GABA^{GAD67+/-}) mice, in which exon 2 (the first coding exon) of the *Gad1* gene has been removed, were obtained from Richard Palmiter (Chattopadhyaya et al., 2007). All animals were euthanized on P56 [n=7 (5 males and 2 females) per genotype]. Brains were processed and tissue was cut as described above for quantification of mRNA levels using in situ hybridization (ISH). In addition, subsequent sections, spanning approximately Bregma +1.10 mm to +0.50 mm, were cut and collected into tubes for protein analysis. Due to tissue loss, only 6 pairs of animals were utilized in the protein study.

For quantitative PCR analysis, due to limited tissue availability, whole frontal cortex sections (Bregma +2.8 mm to +2.1 mm) were utilized. Sections were collected into tubes containing TRIzol reagent (Invitrogen, Carlsbad, CA) (Hashimoto et al., 2008).

In situ hybridization

ISH was performed as described previously (Hashimoto et al., 2005). A 311 base pair fragment corresponding to bases 151–461 of the mouse *Gad1* gene (GAD67) (Genbank accession number Y12257), a 339 base pair fragment corresponding to bases 256–594 of the mouse *Pvalb* gene (PV) (GenBank accession number X59382), and a 462 base pair fragment corresponding to bases 691–1152 of the mouse *Slc6a1* gene (GAT1) (GenBank accession number NM_178703) were amplified by PCR. Nucleotide sequencing revealed 100% homology for the amplified fragments to previously reported sequences. Sense and antisense riboprobes were generated by *in vitro* transcription in the presence of ³⁵S-CTP as previously described (Hashimoto et al., 2005). Following a standard hybridization protocol (Hashimoto et al., 2003), sections from all mice for a given comparison were exposed to BioMax MR film (Kodak, Rochester, NY) for 21 (GAT1), 40 (GAD67), or 72 (PV) hours.

For grain counting of GAD67 and PV mRNAs, slides were then coated with NTB2 emulsion (Kodak) using a mechanical dipper, Auto-dip Emulsion Coater (Ted Pella, Redding, CA), at a constant withdrawal speed and temperature, to ensure the consistency of emulsion thickness across sections. After 10 (GAD67) or 37 (PV) days, slides were exposed at 4°C, developed with D-19 (Kodak), washed, and counterstained using cresyl violet.

All slides for each comparison were contained in a single experimental run and exposed on the same film. Riboprobe specificity was confirmed by an absence of signal with sense probes, the location of grain clusters only over neurons in emulsion-dipped slides, and by the finding that each antisense probe produced the expected laminar pattern of mRNA expression (Hashimoto et al., 2005; Yasumi et al., 1997).

Quantification of in situ hybridization data

To determine total GAD67, PV, and GAT1 mRNA levels, 3 evenly spaced coronal sections [spanning ~144 µm; +1.98 to +1.54 Bregma (Paxinos and Franklin, 2001)] containing primary and secondary motor (M1 and M2) and prefrontal cortex (PFC), including the cingulate and prelimbic cortices, from each animal were quantified as described previously (Hashimoto et al., 2005). Briefly, autoradiographic film images were captured and digitized using a Microcomputer Imaging Device (MCID) system (Imaging Research Inc., Canada) (5.1 µm/pixel resolution). All slides for each comparison were acquired in the same session with identical room illumination, black and gain levels, and flatfield correction. Optical density (OD) levels of GAD67, PV, and GAT1 mRNAs were measured in each hemisphere from the pial surface to the white matter and expressed as nanoCuries per gram (nCi/g) of tissue by reference to ¹⁴C standards (ARC, St. Louis, MO) exposed to the same film. All OD values were corrected by subtracting background measured in the white matter. Separate OD measures were made for M1/M2 and PFC (see Results for rationale). Within a region, measures from each hemisphere were averaged into a single measure per section.

Total GAD67 mRNA levels in GABA^{GAD67+/-} mice have been previously published (Eggan et al., 2012), and were measured using an identical experimental procedure in the same animals utilized in the present study.

To quantify the GAD67 and PV mRNA levels per neuron in the P50 PV^{GAD67+/-} animals, emulsion-dipped and Nissl-counterstained sections were quantified as described previously (Hashimoto et al., 2005). MCID software and a Nikon microscope (Melville, NY) with a motorized stage were used to place 2 (GAD67) or 4 (PV) immediately adjacent columns of sampling boxes (120 µm×170 µm) continuously from the pial surface to the layer 6-white matter border in M1. Sampling circles with a fixed diameter of 16 µm [covering the largest GAD67+ neurons in mouse cortex (Solberg et al., 1988)] were placed over GAD67 or PV silver grain clusters, and the number of silver grains per circle was quantified. All values were corrected by subtracting background grains per cluster measured in a 120 µm×170 µm sampling box placed in the white matter. One pair of animals was not counted for PV due to a damaged slide. A total of 1630 and 1628 (GAD67) and 473 and 496 (PV) grain clusters were analyzed for PV^{GAD67+/+} and PV^{GAD67+/-} mice, respectively.

Quantitative PCR

Total RNA was isolated from TRIzol homogenates, cleaned by RNeasy columns (Qiagen, Valencia, CA), and used to synthesize cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). All primer pairs demonstrated: 1) high amplification efficiency (>96%) across a wide range of cDNA dilutions; 2) specific single products in dissociation curve analysis; and 3) melting temperatures similar to those predicted by oligonucleotide software. Power SYBR Green dye and an ABI StepOne Plus

Real Time PCR System (Applied Biosystems) were used to perform quantitative PCR. The difference in cycle threshold (dCT) for GAD67, PV, and GAT1 mRNA expression was calculated by subtracting the geometric mean of the cycle threshold for the three reference genes (beta actin, NM_007393; cyclophilin A, NM_008907; and glyceraldehyde-3-phosphate dehydrogenase, NM_008084) from the cycle threshold of the mRNA of interest. Because this dCT represents the log₂-transformed expression ratio of the target transcript to the geometric mean of the 3 reference genes, the relative expression level of GAD67 mRNA was determined as 2^{-dCT} (Hashimoto et al., 2008; Vandesompele et al., 2002).

Western blotting

Tissue was homogenized by sonication in an amount of SDS buffer (125mM Tris pH 7, 2% SDS, 10% glycerol) equal to 30 times the tissue volume. Protein extraction and Western blotting were performed as described previously (Curley et al., 2011), using β -tubulin (mouse, 1:20,000, Millipore, Billerica, MA) and GAD67 (goat, 1:800, R&D Systems, Minneapolis, MN) antibodies. In an effort to reduce, and account for, the within- and between-animal measurement variance, replicate measures for each pair of animals were made both within and across gels. Samples from both members of a pair of animals were loaded in adjacent lanes of the same gel. Each pair of samples was loaded 4 times on a gel, and 4 replicate gels were run per pair and processed simultaneously (i.e., 16 replicate measures per animal).

Quantification of Western blotting data

Bands were measured in arbitrary OD units using the Odyssey® Infrared Imaging System software (LI-COR Biosciences, Lincoln, NE) by a single investigator (AAC). OD was measured by drawing a rectangle around each band, and every band was measured 3 times using a new rectangle. Background OD was measured according to LI-COR specifications. To control for differences in the amount of protein loaded per lane, each GAD67 OD measure was corrected by the β -tubulin OD measure from the same lane.

Statistical analyses

In order to control for experimental variance, each wild type animal was paired with an experimental animal, matched for sex and litter, and pairs of animals were processed together in all experiments. Accordingly, paired t tests were used to test the effect of genetic condition on OD measures and number of grains per cluster using mean values across all sections from each animal. Analysis of variance (ANOVA) was used to assess the effect of age on OD in PV^{GAD67+/-} animals. Paired t tests were used to test the effect of genetic condition on normalized GAD67 protein levels using mean values across all of the gels from each animal. Since GAD67 levels were genetically manipulated to be lower in both models, all reported p values for GAD67 are one-tailed. All other p values are two-tailed.

Results

Transcript levels in PV^{GAD67+/-} mice

Most postmortem studies of schizophrenia to date have examined tissue-level expression of GAD67, PV, and GAT1 mRNAs, and thus similar measures were made in PV^{GAD67+/-} mice in order to maximize comparisons to the human disease. The majority of GAD67 measures in schizophrenia have been made in PFC; however, unlike in humans, PFC levels of PV in the mouse are low relative to other cortical regions such as M1/M2 (Hashimoto et al., 2005), likely a consequence of the presence of significantly fewer PV neurons in mouse PFC (Fitzgerald et al., 2011). Since Cre expression, and therefore GAD67 expression, is driven by the endogenous PV promoter in PV^{GAD67+/-} mice, PFC may not be a sensitive region for

detecting changes in gene expression in mouse PV neurons. Maximizing sensitivity is especially important given that PV^{GAD67+/-} mice exhibit lower GAD67 only in PV neurons. Thus, we first examined mRNA levels in M1/M2, homologous to primary motor cortex where alterations in GAD67, PV, and GAT1 mRNAs are prominent in schizophrenia (Hashimoto et al., 2008). Mean ($\pm$ SD) GAD67 mRNA levels were significantly 17.4% lower ($t_5=2.23$, $p=0.038$) in M1/M2 of P50 PV^{GAD67+/-} mice (384 ± 60) relative to PV^{GAD67+/+} mice (465 ± 60) (Figure 1B). PV^{GAD67+/-} mice are missing one allele from every PV neuron and PV neurons comprise approximately 50% of all GABA interneurons in the mouse cortex. Thus, the genetic manipulation of a 50% reduction in 50% of GABA neurons should produce a 25% reduction in GAD67 mRNA, similar to the 17% we observed. However, contrary to our hypothesis, neither PV ($t_5=1.03$, $p=0.350$) nor GAT1 ($t_5=-0.31$, $p=0.769$) mRNA levels significantly differed between PV^{GAD67+/-} mice and PV^{GAD67+/+} mice in M1/M2 (Figure 1B).

Consistent with the idea that the PFC is a less sensitive region for detecting changes in PV neurons, GAD67 mRNA levels were nonsignificantly 8.7% lower ($t_5=1.18$, $p=0.146$) in P50 PV^{GAD67+/-} mice relative to PV^{GAD67+/+} mice (Figure 1C). Similar to M1/M2, neither PV mRNA nor GAT1 mRNA levels in the PFC were significantly different between P50 PV^{GAD67+/+} and PV^{GAD67+/-} mice (PV: $t_5=1.45$, $p=0.208$; GAT1: $t_5=0.60$, $p=0.576$) (Figure 1C). Based on the more robust deficit of GAD67 mRNA in M1/M2, all subsequent measures in PV^{GAD67+/+} and PV^{GAD67+/-} mice were made in this cortical region.

In order to assess whether developmental compensations might account for the lack of change in PV and GAT1 in the P50 PV^{GAD67+/-} mice, we also examined two younger age groups of mice (P17 and P30). PV^{GAD67+/+} mice exhibited the predicted developmental trajectories for GAD67, PV, and GAT1 mRNAs in M1/M2 (Kiser et al., 1998; de Lecea et al., 1995; Xia et al., 1993). Across development, GAD67 mRNA levels increased significantly by 135% ($F_{1,14}=6.93$, $p=0.008$) and PV mRNA levels increased significantly by 241% ($F_{1,14}=56.00$, $p<0.001$). GAT1 mRNA levels decreased by 10% between P17 and P50 ($F_{1,14}=3.94$, $p=0.044$), a much smaller change than GAD67 or PV, consistent with previous studies (Kiser et al., 1998; de Lecea et al., 1995; Xia et al., 1993).

GAD67 mRNA levels exhibited similar magnitudes of reduction in PV^{GAD67+/-} mice at all three ages examined (Figure 2A), suggesting that the GAD67 deletion was largely completed by P17. In P17 animals, GAD67 mRNA was 15.8% lower in PV^{GAD67+/-} mice relative to PV^{GAD67+/+} mice, although this difference did not achieve statistical significance ($t_4=1.81$, $p=0.072$). In P30 animals, GAD67 mRNA was significantly 17.7% lower ($t_5=6.33$, $p=0.001$) in PV^{GAD67+/-} mice (333 ± 50 nCi/g) relative to PV^{GAD67+/+} mice (405 ± 58 nCi/g).

Similar to the results at P50, PV mRNA levels in M1/M2 were not significantly different between PV^{GAD67+/-} and PV^{GAD67+/+} mice at P17 ($t_4=-0.37$, $p=0.730$) or P30 ($t_5=-2.56$, $p=0.051$) (Figure 2B). GAT1 mRNA levels were also not significantly different between PV^{GAD67+/-} mice and PV^{GAD67+/+} mice at either P17 ($t_4=-0.42$, $p=0.700$) or P30 ($t_5=0.71$, $p=0.507$) (Figure 2C).

The lack of a change in PV mRNA in PV^{GAD67+/-} mice could be due to an insensitivity of the film analyses, and thus we quantified PV mRNA levels per neuron. The mean number of PV grains per neuron was not significantly different between PV^{GAD67+/-} and PV^{GAD67+/+} mice in M1/M2 ($t_4=-0.77$, $p=0.483$) (right panel of Figure 3B), suggesting that the lack of change in PV mRNA at the film level was not due to insufficient sensitivity. In contrast to the PV mRNA levels per neuron, the mean number of GAD67 grains per neuron was 30.5% lower ($t_5=9.55$, $p<0.001$) in PV^{GAD67+/-} mice (44 ± 6) relative to PV^{GAD67+/+} mice (63 ± 9) (left panel of Figure 3B). These data confirm that the genetic manipulation did produce the

expected reduction in GAD67 mRNA, and that the cell type-specific deficit is more apparent when examined at the cellular level. Since GAT1 is present in both PV and non-PV cells that presumably contain normal levels of GAD67 mRNA, it would be difficult to detect a difference in only a subpopulation of GAT1 mRNA-containing cells. Thus, cellular levels of GAT1 mRNA were not quantified.

Transcript levels in GABA^{GAD67+/-} mice

In PV^{GAD67+/-} mice, GAD67 is reduced specifically in PV neurons beginning on approximately P10–P14, when PV mRNA expression turns on in the murine neocortex (del Rio et al., 1994; Meyer et al., 2002). Given the lack of change in PV and GAT1 in PV^{GAD67+/-} mice, we also examined these transcripts in a more pronounced and earlier model of GAD67 reduction, P56 germline GABA^{GAD67+/-} mice, in which tissue levels of GAD67 mRNA are reduced in all GABA neurons during early prenatal development (Eggan et al., 2012). In M1/M2, GAD67 mRNA levels were significantly 36.6% lower ($t_6=7.89$, $p<0.001$) in GABA^{GAD67+/-} (872 ± 78 nCi/g) compared to GABA^{GAD67+/+} (1375 ± 111 nCi/g) mice (Figure 4B). In contrast, neither PV ($t_6=-1.03$, $p=0.344$) nor GAT1 ($t_6=-0.36$, $p=0.731$) mRNA levels in M1/M2 differed between GABA^{GAD67+/+} and GABA^{GAD67+/-} mice (Figure 4B).

In order to exclude the possibility that the lack of change in PV and GAT1 mRNAs is region-specific, we also examined these transcripts in the PFC. Similar to the findings in M1/M2, GAD67 mRNA was significantly 37.2% lower ($t_6=7.67$, $p<0.001$; data from Eggan et al., 2012) in the PFC of GABA^{GAD67+/-} mice, but neither PV ($t_6=0.33$, $p=0.754$) nor GAT1 ($t_6=1.32$, $p=0.235$) mRNAs differed between GABA^{GAD67+/-} and GABA^{GAD67+/+} mice (Figure 4C).

In order to confirm the ISH findings, we analyzed GAD67, PV, and GAT1 mRNA levels by quantitative PCR. Similar to the ISH data, levels of GAD67 mRNA were significantly 25.5% lower ($t_6=3.25$, $p=0.009$) in whole frontal cortex of GABA^{GAD67+/-} mice (0.080 ± 0.03) relative to GABA^{GAD67+/+} mice (0.107 ± 0.04), but neither PV ($t_6=-0.989$, $p=0.361$) nor GAT1 ($t_6=-0.574$, $p=0.587$) mRNA levels differed between the genotypes.

Protein levels in GABA^{GAD67+/-} mice

In order to confirm that lower GAD67 mRNA expression resulted in less translation of the cognate protein, we used Western blotting to quantify GAD67 protein levels in brain tissue homogenates (spanning Bregma +1.10 to +0.50) from GABA^{GAD67+/-} mice. GAD67 protein levels were significantly 27.0% lower ($t_5=7.22$, $p<0.001$) in GABA^{GAD67+/-} (0.80 ± 0.21) compared to GABA^{GAD67+/+} (1.09 ± 0.26) mice (Figure 5B). These data are consistent with a previous report (Chattopadhyaya et al., 2007), and confirm that the genetic manipulation extends to the protein level.

Discussion

The goal of our study was to test in murine models whether the strong positive associations of deficient cortical levels of GAD67 with PV and GAT1 mRNAs in schizophrenia could represent a cause-and-effect molecular mechanism in the illness. That is, that PV and GAT1 mRNAs are down-regulated in schizophrenia as compensatory responses to deficient GAD67 expression and GABA synthesis. The measurements of these transcripts in the murine models in a manner identical to those used in the human illness enhanced the translational testing of the hypothesized molecular mechanism. We found that GAD67 mRNA was significantly reduced by ~17% at the tissue level across development and by 31% at the cellular level in adult PV^{GAD67+/-} mice, and that tissue-level GAD67 mRNA and

protein levels were significantly reduced by 37% and 27%, respectively, in adult GABA^{GAD67+/-} mice. However, neither PV nor GAT1 mRNA levels were altered in either mouse model at the tissue level in either M1/M2 or PFC, regardless of the magnitude, cell type-specificity or developmental age of the deficit in GAD67 mRNA expression. Moreover, PV was not significantly altered at the cellular level in PV^{GAD67+/-} mice. In combination, these findings do not support the prevailing hypothesis that reduced GAD67 expression is “upstream” of lower levels of PV and GAT1 mRNAs in schizophrenia (Lewis et al., 2005).

Several factors suggest that these findings are unlikely to represent false negatives. First, the use of both tissue and cellular levels of analysis, and both ISH and quantitative PCR methods, provides a thorough and sensitive quantification of mRNA expression levels. Second, although we cannot exclude the possibility of subtle changes in PV and GAT1 expression in either murine model, the sample size employed had 99.6% and 91.8% power, respectively, to detect the average 20% and 18% reductions in PV and GAT1 mRNA reported in schizophrenia (Fung et al., 2010; Ohnuma et al., 1999; Hashimoto et al., 2008; Volk et al., 2012). Third, although compensatory increases in the other GABA synthesizing enzyme, GAD65, could have resulted in normal GABA levels in the GAD67-deficient mice, the following lines of evidence make this scenario unlikely: 1) prior studies of GABA^{GAD67+/-} mice reported no change in GAD65 mRNA (Asada et al., 1997) or protein (Chattopadhyaya et al., 2007) levels; 2) the presence of reduced IPSC amplitude and faster IPSC decay time, as well as pyramidal cell hyperactivity, in P30 PV^{GAD67+/-} mice are all consistent with lower GABA function (Lazarus and Huang, 2010); 3) recent data indicate that some neurons use mainly GAD67 and others rely principally on GAD65 (Fish et al., 2011), consistent with other findings that GABA neurons do not use GAD65 and GAD67 interchangeably (Battaglioli et al., 2003).

The likelihood of false negative findings is also reduced by the use of two mouse models that differ in their magnitudes of GAD67 reduction. The mean GAD67 mRNA deficit at the tissue level in the PV^{GAD67+/-} mice (-17%) is very similar to that observed in the largest published study of schizophrenia subjects (-15%) (Curley et al., 2011), suggesting that these mice appropriately recapitulate this aspect of the illness. Nevertheless, the finding that GAD67 mRNA levels are not detectable (though the true level of reduction is unknown) in approximately 50% of PV neurons in schizophrenia (Hashimoto et al., 2003) may indicate that the GAD67 mRNA deficit per neuron in schizophrenia is greater than the 31% reduction observed in the PV^{GAD67+/-} mice. However, in the GABA^{GAD67+/-} mouse model that has an even greater tissue-level reduction in GAD67 than that present in schizophrenia, no change in PV and GAT1 mRNA levels were observed. The interpretation that an insufficient reduction in GAD67 does not underlie the negative PV and GAT1 results in these mice is supported by the report that other transcript alterations present in schizophrenia were found in GABA^{GAD67+/-} mice (Eggen et al., 2012).

Although when the GAD67 deficit appears in schizophrenia is unknown, it may occur quite early in development given that cognitive disturbances can be detected in children years before the onset of psychosis (Reichenberg et al., 2010; Niendam et al., 2003). Both genes and the environment are thought to play a role in the pathogenesis of schizophrenia. Thus, lower GAD67 resulting from genetic factors might occur during early prenatal development, whereas an environmental insult, such as complications during late pregnancy (Cannon et al., 2002), would produce a deficit later. In the present study, we modeled these two possible time points of lower GAD67 expression by using GABA^{GAD67+/-} mice in which the reduction in GAD67 expression occurs early in prenatal development and PV^{GAD67+/-} mice which do not exhibit lower GAD67 until the postnatal period [P10-P14, corresponding to roughly the third trimester of gestation in humans (Clancy et al., 2001)]. Our data suggest

that PV and GAT1 mRNAs are not reduced regardless of whether the GAD67 deficit is present from the birth of GABA neurons or not until later in development. However, it is possible that GAD67 is not reduced until a later stage of development in schizophrenia. Thus, reducing GAD67 very early in development, as we have done in the present study, may evoke different effects than would be seen if the reduction occurs during childhood in individuals with schizophrenia.

Although our study provides a robust proof-of-concept test of a human disease mechanism in mice, species differences temper the translational strength of findings in mice. That is, PV^{GAD67+/-} mice may not appropriately model the disease process of schizophrenia due to differences in cortical PV cells between rodents and primates. For example, although the onset of PV expression occurs postnatally in rodents and primates (de Lecea et al., 1995; Hoftman and Lewis, 2011), the timing of onset in rodents actually corresponds to prenatal development in humans (Clancy et al., 2001). In addition, PV cells differ in some physiological properties between rodents and primates (Povysheva et al., 2008). Finally, the proportion of cortical GABA neurons that express PV is about two times greater in rodents than primates (Conde et al., 1994; Tamamaki et al., 2003). Therefore, it is possible that reduced GAD67 expression could produce different compensatory effects in rodent and primate PV cells.

The results of the present study do not support a prevailing hypothesis that lower GAD67 mRNA results in compensatory reductions in PV and GAT1 mRNAs in schizophrenia and suggest the need to consider other explanations for the strong positive correlations among these transcripts in schizophrenia. First, lower levels of PV and GAT1 could be upstream of lower GAD67 expression in schizophrenia. Lower levels of PV increase GABA release (Collin et al., 2005; Vreugdenhil et al., 2003) and the resulting increase in extracellular GABA might evoke a compensatory reduction in GABA synthesis via downregulation of GAD67 expression. Likewise, a deficit in GABA reuptake may result in increased GABA availability and a compensatory downregulation of GABA synthesis. However, GAT1 knockout mice actually exhibit increased GAD67 (Bragina et al., 2008). Second, strong positive correlations among the deficits in GAD67, PV, and GAT1 mRNAs in schizophrenia could also be consistent with the idea that all three transcripts are downstream consequences of some genetic and/or environmental factors that give rise to the illness. Such factors may have their effects mediated by processes that regulate the expression of GABA-related genes. For example, lower signaling through NMDA receptors, which is thought to occur in schizophrenia (Coyle, 2006), has been associated with lower cortical levels of GAD67 and PV mRNAs in some but not all model systems (Behrens et al., 2007; Bullock et al., 2009; Belforte et al., 2010; Benneyworth et al., 2011). Alternatively, altered expression of a transcription factor (Lhx6) that regulates PV neuron development could drive lower GABA-related transcript expression, at least in a subset of individuals with schizophrenia (Volk et al., 2012).

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Disclosure

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Highlights

- PV and GAT1 mRNA are not altered in mice (PV^{GAD67+/-}) with lower GAD67 in PV neurons.
- Cellular analysis confirms the lack of altered PV mRNA levels in the PV^{GAD67+/-} mice.
- PV and GAT1 mRNA are not altered in mice with a germline reduction in GAD67.
- Deficits in PV and GAT1 in schizophrenia may not be the result of lower GAD67.

Figure 1. GAD67, but not PV or GAT1, mRNA levels are reduced in $PVGAD67^{+/-}$ mice

A: representative film autoradiograms illustrating the expression of GAD67, PV, and GAT1 mRNA in the frontal lobe of P50 $PVGAD67^{+/+}$ mice. The density of hybridization signal is presented in pseudocolor according to the calibration bars. The white contours denote the quantified regions in M1/M2 and PFC. Note that PV mRNA expression is much lower in PFC than in M1/M2. B: comparison of M1/M2 GAD67, PV, and GAT1 mRNA levels by film OD in P50 $PVGAD67^{+/+}$ and $PVGAD67^{+/-}$ mice (GAD67: $t_5=2.23$, $p=0.038$; all others, $p=0.350$). C: comparison of PFC GAD67, PV, and GAT1 mRNA levels by film OD in P50 $PVGAD67^{+/+}$ and $PVGAD67^{+/-}$ mice (all transcripts, $p=0.146$). Mean values for each group are indicated by hash marks.

Figure 2. GAD67, but not PV or GAT1, mRNA levels are reduced in PV^{GAD67+/-} mice across development

Comparison of GAD67 (A), PV (B), and GAT1 (C) mRNA levels by film OD in PV^{GAD67+/+} and PV^{GAD67+/-} mice (GAD67 P30: $t_5=6.33$, $p=0.001$; GAD67 P50: $t_5=2.23$, $p=0.038$; all others, $p=0.051$). Mean values for each condition are indicated by hash marks.

Figure 3. Cellular levels of GAD67, but not PV, mRNA are reduced in PV^{GAD67+/-} mice
 A: representative micrographs of GAD67 grains in P50 PV^{GAD67+/+} (left) and PV^{GAD67+/-} (right) mice. B: comparison of GAD67 (left) and PV (right) mRNA levels in M1 by grain counting in litter-matched pairs of P50 PV^{GAD67+/+} and PV^{GAD67+/-} mice (GAD67: $t_5=9.55$, $p<0.001$; PV: $p=0.483$). Mean values for each condition are indicated by hash marks.

Figure 4. GAD67, but not PV or GAT1, mRNA levels are reduced in $GABA^{GAD67+/-}$ mice

A: representative film autoradiograms illustrating the expression of GAD67, PV, and GAT1 mRNA in the frontal lobe of P56 $GABA^{GAD67+/+}$ mice. The density of hybridization signal is presented in pseudocolor according to the calibration bars. The white contours denote the quantified regions in M1/M2 and PFC. B: comparison of GAD67, PV, and GAT1 mRNA levels in M1/M2 by film OD in P56 $GABA^{GAD67+/+}$ and $GABA^{GAD67+/-}$ mice (GAD67: $t_6=7.89$, $p<0.001$; all others: $p=0.344$). C: comparison of GAD67, PV, and GAT1 mRNA levels in PFC by film OD of P56 $GABA^{GAD67+/+}$ and $GABA^{GAD67+/-}$ mice [GAD67: $t_6=7.67$, $p<0.001$; all others: $p=0.235$]. Mean values for each group are indicated by hash marks. PFC GAD67 OD levels are from Eggen et al., 2012.

Figure 5. GAD67 protein levels are reduced in GABA^{GAD67+/-} mice

A: lanes from a representative Western blot loaded with brain tissue homogenate (spanning Bregma +1.10 to +0.50) from a GABA^{GAD67+/+} and GABA^{GAD67+/-} litter-matched pair. B: comparison of tubulin-normalized GAD67 protein levels in pairs of P56 GABA^{GAD67+/+} and GABA^{GAD67+/-} mice ($t_5=7.22$, $p<0.001$). Mean values for each condition are indicated by hash marks.