Cortical Gene Expression After a Conditional Knockout of 67 kDa Glutamic Acid Decarboxylase in Parvalbumin Neurons

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In the cortex of subjects with schizophrenia, expression of glutamic acid decarboxylase 67 (GAD67), the enzyme primarily responsible for cortical GABA synthesis, is reduced in the subset of GABA neurons that express parvalbumin (PV). This GAD67 deficit is accompanied by lower cortical levels of other GABA-associated transcripts, including GABA transporter-1, PV, brain-derived neurotrophic factor (BDNF), tropomyosin receptor kinase B, somatostatin, GABA$_A$ receptor α1 subunit, and KCNS3 potassium channel subunit mRNAs. In contrast, messenger RNA (mRNA) levels for glutamic acid decarboxylase 65 (GAD65), another enzyme for GABA synthesis, are not altered. We tested the hypothesis that this pattern of GABA-associated transcript levels is secondary to the GAD67 deficit in PV neurons by analyzing cortical levels of these GABA-associated mRNAs in mice with a PV neuron-specific GAD67 knockout. Using in situ hybridization, we found that none of the examined GABA-associated transcripts had lower cortical expression in the knockout mice. In contrast, PV, BDNF, KCNS3, and GAD65 mRNA levels were higher in the homozygous mice. In addition, our behavioral test battery failed to detect a change in sensorimotor gating or working memory, although the homozygous mice exhibited increased spontaneous activities. These findings suggest that reduced GAD67 expression in PV neurons is not an upstream cause of the lower levels of GABA-associated transcripts, or of the characteristic behaviors, in schizophrenia. In PV neuron-specific GAD67 knockout mice, increased levels of PV, BDNF, and KCNS3 mRNAs might be the consequence of increased neuronal activity secondary to lower GABA synthesis, whereas increased GAD65 mRNA might represent a compensatory response to increase GABA synthesis.

Key words: schizophrenia/cerebral cortex/mouse/in situ hybridization/prepulse inhibition/working memory

Introduction

In the cerebral cortex of subjects with schizophrenia, the 67 kDa isoform of glutamic acid decarboxylase (GAD67), the enzyme principally responsible for cortical γ-amino butyric acid (GABA) synthesis, is markedly reduced in the subset of GABA neurons that express parvalbumin (PV), indicating altered GABA neurotransmission by these neurons.[1,2] The reduced GAD67 expression in schizophrenia is accompanied by lower cortical expression of other genes associated with GABA neuron functions. For example, reduced messenger RNA (mRNA) and protein levels were observed for GABA transporter-1 (GAT-1) and PV in PV neurons.[1,3–5] Reduced mRNA levels were also reported for 2 proteins that mediate trophic effects on GABA neurons, namely brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase B (TrkB),[6–8] for somatostatin (SST),[9–11] a specific marker for another subset of cortical GABA neurons, for GABA$_A$ receptor (GABA$_A$R) α1 subunit,[12,13] and for KCNS3 voltage-gated potassium channel subunit,[14,15] which appears to be important for electrophysiological properties of PV neurons.[14,16,17] In contrast, cortical mRNA levels for glutamic acid decarboxylase 65 (GAD65), another enzyme for GABA synthesis, appear to be unaffected in schizophrenia.[18–20]

Reduced GAD67 expression in PV neurons could induce changes in other GABA-associated transcripts. For example, the resulting reduced synaptic availability of GABA could induce downregulation of GAT-1, PV, and KCNS3 expression which would serve as compensatory responses by reducing presynaptic GABA reuptake,[21] increasing GABA release,[22] and enhancing PV neuron excitability,[16] respectively. Reduced GABA neurotransmission by PV neurons also might lead to excessive pyramidal neuron activity which could then trigger

a compensatory reduction in dendritic spines during postnatal development.[23,24] Following maturation, the resulting pyramidal neuron hypoactivity could lead to an activity-dependent downregulation of BDNF, TrkB, and GABA$_A$R α1 subunit expression[25–27] in these neurons. Finally, lower BDNF signaling could drive down SST mRNA expression,[9,10,28] which might provide a compensatory disinhibition of PV neurons by SST neurons, as SST neurons suppress PV neuron activity.[29,30] Consistent with this interpretation, the reduction in GAD67 mRNA levels in schizophrenia was significantly correlated with the reductions in GAT-1,[31] PV,[1] BDNF,[7] TrkB,[7] and SST[9,13] mRNAs.

Based on these findings, we hypothesized that lower levels of certain GABA-associated transcripts in the cortex of schizophrenia subjects could be secondary to the GAD67 deficit in PV neurons. In order to test this hypothesis, we created a conditional knockout mouse line in which *Gad1*, the gene encoding GAD67, was selectively inactivated in PV neurons. In the cortex of these mice, we assessed mRNA levels for GAT-1, PV, KCNS3, BDNF, TrkB, GABA$_A$R α1, SST, and GAD65. We also tested whether the GAD67 knockout in PV neurons resulted in behavioral alterations characteristic of patients with schizophrenia by subjecting the mutant mice to a battery of behavioral tests. As GAD67 mRNA expression is undetectable in a subset of GABA neurons,[32,33] including ~50% of PV neurons,[1] in schizophrenia, we were particularly interested in the homozygous knockout that recapitulates such profound GAD67 deficits in PV neurons.

Methods

PV Neuron-Specific Inactivation of the Gad1 Gene

In order to achieve a PV neuron-specific inactivation of *Gad1*, which codes for GAD67, we used 2 genetically engineered mouse lines. The first line contained an IRES-Cre-pA cassette inserted into the 3′-untranslated region of exon 5 of the *Pvalb* gene that encodes PV (PV-Cre mice).[34] The second line contained floxed exon 1 of the *Gad1* gene (floxed-GAD67 mice).[35] Both lines had a mixed genetic background of 129/OlaHsd and C57BL/6. We first crossed homozygous PV-Cre mice (*Pvalb*$^{Cre/Cre}$) and homozygous floxed-GAD67 mice (*Gad1*$^{loxp/loxp}$) to obtain heterozygous mice for both *Pvalb* and *Gad1* genes (*Pvalb*$^{Cre/+}$; *Gad1*$^{loxp/+}$). Mating of these heterozygous mice generated homozygous PV-Cre/heterozygous floxed-GAD67 mice (*Pvalb*$^{Cre/Cre}$; *Gad1*$^{loxp/+}$) and heterozygous floxed-GAD67 mice (*Pvalb*$^{+/+}$;*Gad1*$^{loxp/+}$). We then crossed these 2 genotypes and obtained littermates of 3 genotypes, *Pvalb*$^{Cre/+}$;*Gad1*$^{+/+}$, *Pvalb*$^{Cre/+}$;*Gad1*$^{loxp/+}$, and *Pvalb*$^{Cre/+}$;*Gad1*$^{loxp/loxp}$, and subsequently referred to as control (PV$^{GAD67+/+}$), heterozygous (PV$^{GAD67+/−}$), and homozygous (PV$^{GAD67−/−}$) mice, respectively. Three same sex littermates with different genotypes, PV$^{GAD67+/+}$, PV$^{GAD67+/−}$, and PV$^{GAD67−/−}$, respectively, were designated as a triad. Five triads were used for in situ hybridization analysis at each of 8 and 15 weeks of age and for Western blot analysis at 8 weeks. Behavioral tests were performed between 11 and 15 weeks with 3 mice from 1 triad tested on the same day. Four triads were used for the rotarod test. Six triads were used in the open field, light–dark transition, prepulse inhibition, and Y-maze tests. The animal experiments were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and approved by the Committee on Animal Experimentation of Kanazawa University.

Tissue Preparation and Single-Label in situ Hybridization With ^{35}S-Labeled Riboprobes

Serial coronal sections (12 μm) were cut from +1.94 mm to +1.54 mm from the bregma, and 3 sections evenly spaced at ≈160 μm intervals were selected from each mouse and subjected to in situ hybridization (ISH) for each mRNA of interest. Riboprobe synthesis and hybridization were performed as described previously[15] and in the [supplementary material](). Hybridized sections were exposed to BioMax MR film (Kodak) for 2–7 days depending on the abundance of transcripts. Throughout these ISH procedures, sections from each triad were processed together.

All antisense riboprobes revealed distinctive signal distributions in the mouse cortex that were consistent with previous ISH studies[7,9,36–38] and Allen Brain Atlas (http://mouse.brain-map.org/). No signal beyond the background was detected with sense riboprobes for all mRNAs.

Quantification of mRNA Expression Levels

Trans-illuminated autoradiographic film images were captured by a video camera, digitized and analyzed using a Microcomputer Imaging Device (MCID) system (InterFocus Imaging Ltd). Images of adjacent sections stained with cresyl violet were also captured and superimposed onto the autoradiographic images to draw contours of the pia mater and gray matter/white matter border. Quantification of mRNA levels was done in 2 regions of interest: the prefrontal cortex (PFC), including the cingulate and prelimbic cortices, and the sensorimotor cortex (SMC), including the primary sensory and primary motor cortices.[39] Optical densities in these regions were expressed as microcuries per gram of tissue (μCi/g) by reference to Carbon-14 radioactive standards (ARC Inc) exposed on the same film. The data were averaged across the 3 sections in each animal.

Dual-Label ISH and Grain Analysis

To directly verify reduced GAD67 mRNA expression in PV neurons in the knockout mice at 8 weeks of age,

we performed dual-label ISH with ^{35}S-labeled riboprobe for GAD67 mRNA and digoxigenin (DIG)-labeled riboprobe for PV mRNA as described previously[15] and then carried out grain analysis to quantify relative GAD67 mRNA levels in PV neurons in the SMC of PV$^{GAD67+/+}$, PV$^{GAD67+/-}$, and PV$^{GAD67-/-}$ mice. Procedures for dual-label ISH and grain analysis are described in the supplementary material.

Western Blotting Analysis

GAD67 protein levels were quantified in the SMC by Western blotting analysis using anti-GAD67 and anti-β-tubulin antibodies. Protein extraction and subsequent Western blotting analysis were performed as described in the supplementary material.

Behavioral Tests

Influences of the PV neuron-specific GAD67 knockout on motor coordination and learning, general activity, anxiety, sensorimotor gating, and working memory were tested in a behavioral test battery that consists of rotarod, open field, light–dark transition, prepulse inhibition (PPI), and Y-maze tests. Procedures and equipment for each test are described in the supplementary material.

Statistical Analyses

Analyses were performed on SPSS (SPSS, Inc). Across the 3 genotypes (PV$^{GAD67+/+}$, PV$^{GAD67+/-}$, and PV$^{GAD67-/-}$), differences in cortical mRNA and protein expression levels, as well as behavioral measures in the open field, light–dark transition, and Y-maze tests were assessed with single-factor ANOVA models. Genotype effects on behavioral data obtained from the rotarod and PPI tests were analyzed with 2-factor ANOVA models with repeated measures in 1 factor (trial for rotarod test and prepulse intensity for PPI test). If the ANOVA models detected significance, Tukey's post hoc tests were used to identify statistically different genotypes. All statistical tests were conducted with an α-level = .05.

Results

GAD67 Knockout in Cortical PV Neurons

Single-label ISH with ^{35}S-labeled riboprobe revealed reduced GAD67 mRNA expression in both the PFC and SMC of mice with a PV neuron-specific *Gad1* inactivation compared with control mice at both 8 and 15 weeks of age (figure 1A). The ANOVA model detected significant effects of genotype on the mRNA levels in both regions at 8 weeks ($F_{2,12}$ = 12.92, P = .001 and $F_{2,12}$ = 31.06, P < .001 for the PFC and SMC, respectively) and at 15 weeks ($F_{2,12}$ = 4.43, P = .036 and $F_{2,12}$ = 7.76, P = .007 for the PFC and SMC, respectively; figure 1A). At 8 weeks, GAD67 mRNA levels in the homozygous (PV$^{GAD67-/-}$)

mice were significantly decreased by 13.8% and 30.2% in the PFC and SMC, respectively, compared with control (PV$^{GAD67+/+}$) mice (figure 1A). At 15 weeks, the mRNA levels in PV$^{GAD67-/-}$ mice were significantly decreased by 22.0% and 27.1% in the PFC and SMC, respectively, compared with PV$^{GAD67+/+}$ mice (figure 1A). In the heterozygous (PV$^{GAD67+/-}$) mice, GAD67 mRNA levels were similar to PV$^{GAD67-/-}$ mice at 8 weeks and intermediate between PV$^{GAD67-/-}$ and PV$^{GAD67+/+}$ mice at 15 weeks (figure 1A). In the Western blotting analysis, we detected a gene dose-dependent effect of the *Gad1* inactivation on GAD67 protein levels ($F_{2,12}$ = 34.92, P < .001) in the SMC at 8 weeks (figure 1B). Compared with PV$^{GAD67+/+}$ mice, GAD67 protein levels were significantly decreased by 16.6% and 39.8% in PV$^{GAD67+/-}$ and PV$^{GAD67-/-}$ mice, respectively (figure 1B). Finally, with dual-label ISH that detected GAD67 and PV mRNAs as silver grain accumulation and color reaction, respectively (figure 1C), we found that GAD67 mRNA levels in individual SMC PV neurons differed significantly by genotype ($F_{2,12}$ = 60.58, P < .001) at 8 weeks of age. Compared with PV$^{GAD67+/+}$ mice, mean grain densities in PV neurons were significantly decreased by 48.5% and 79.4% in PV$^{GAD67+/-}$ and PV$^{GAD67-/-}$ mice, respectively (figure 1D). In contrast, grain density appeared similar across the 3 genotypes in non-PV GABA neurons (figure 1C). These findings indicate that reductions in GAD67 expression in PV neurons, especially in the homozygous mice, were robust and similar to those present in schizophrenia.[1]

Expression of GABA-Associated Transcripts

For each GABA-associated transcript examined, the cortical expression pattern did not differ across PV$^{GAD67+/+}$, PV$^{GAD67+/-}$, and PV $^{GAD67-/-}$ mice (figure 2). The ANOVA model and subsequent post-hoc tests revealed significant increases in cortical mRNA levels for PV, KCNS3, BDNF, and GAD65 in PV$^{GAD67-/-}$ mice, relative to age- and sex-matched PV$^{GAD67+/+}$ littermates (figure 3, table 1). For both PV and GAD65 mRNAs, significant increases were detected in the SMC at 8 and 15 weeks (table 1, figures 3B and 3H). KCNS3 mRNA levels were significantly greater in the PFC and SMC at 8 weeks (table 1, figure 3C). BDNF mRNA levels were increased in the PFC and SMC at 8 weeks and in the SMC at 15 weeks (table 1, figure 3D). None of the other GABA-associated transcripts (ie, GAT-1, TrkB, GABA$_A$R α1, or SST), whose cortical expression levels are lower in schizophrenia subjects, showed a significant reduction in their expression levels in the cortex of PV neuron-specific GAD67 knockout mice.

Behavioral Phenotype of Mice With GAD67 Knockout in PV Neurons

In the rotarod test, PV$^{GAD67+/+}$, PV$^{GAD67+/-}$, and PV$^{GAD67-/-}$ mice all exhibited similar motor coordination and learning. The repeated measures ANOVA model did not detect
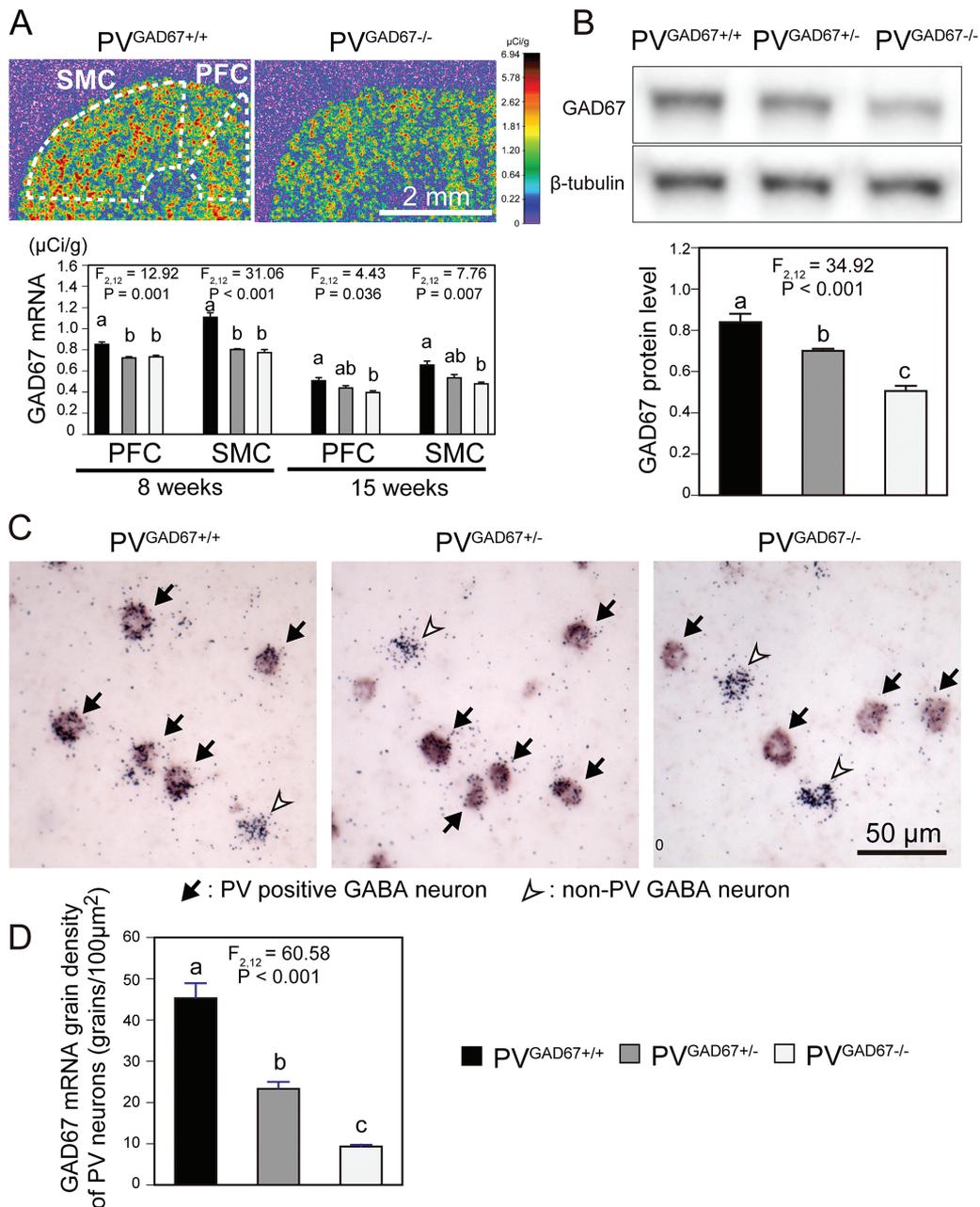
Fig. 1. Glutamic acid decarboxylase 67 (GAD67) knockout in cortical parvalbumin (PV) neurons. A: (Top) Film images of cortical GAD67 messenger RNA (mRNA) expression, detected by in situ hybridization (ISH), in a PV^GAD67+/+ (left) and a PV^GAD67−/− (right) mice from the same triad. The optical densities were presented in a pseudo-color manner according to the color bar on the right. Quantification was performed for the prefrontal cortex (PFC) and sensorimotor cortex (SMC) within the areas contoured by broken lines. (Bottom) Mean ± SEM GAD67 mRNA expression levels in the PFC and SMC of PV^GAD67+/+ (black bars), PV^GAD67+/− (gray bars), and PV^GAD67−/− (white bars) mice at 8 and 15 weeks of age. Bars not sharing the same letter are statistically different. B: (Top) Immunoreactive bands for GAD67 and β-tubulin, detected by Western blotting, in the SMC of a triad of PV^GAD67+/+, PV^GAD67+/−, and PV^GAD67−/− mice at 8 weeks of age. (Bottom) Mean ± SEM β-tubulin-normalized GAD67 protein levels in the SMC of PV^GAD67+/+, PV^GAD67+/−, and PV^GAD67−/− mice at 8 weeks of age. C: Photomicrographs of dual-label ISH detecting GAD67 mRNA signals as silver grain clusters and PV mRNA signals as color reaction products. In the PFC of 8-week-old PV^GAD67+/+ (left), PV^GAD67+/− (center), and PV^GAD67−/− (right) mice, GAD67 mRNA expression is detected in PV neurons (arrows), as well as in other GABA neurons that are indicated by silver grain clusters without color reaction (white arrowhead). D: Mean ± SEM grain densities of individual PV neurons in the SMC of PV^GAD67+/+, PV^GAD67+/−, and PV^GAD67−/− mice at 8 weeks of age.

a significant effect of genotype ($F_{2,9} = 0.027$, $P = .973$) or genotype × trial interaction ($F_{2,9} = 0.56$, $P = .590$) on the latency to fall from the accelerating rotarod. The open field and light-dark transition tests indicated that the PV neuron-specific GAD67 knockout had little influence on anxiety, as genotype did not have a significant effect on
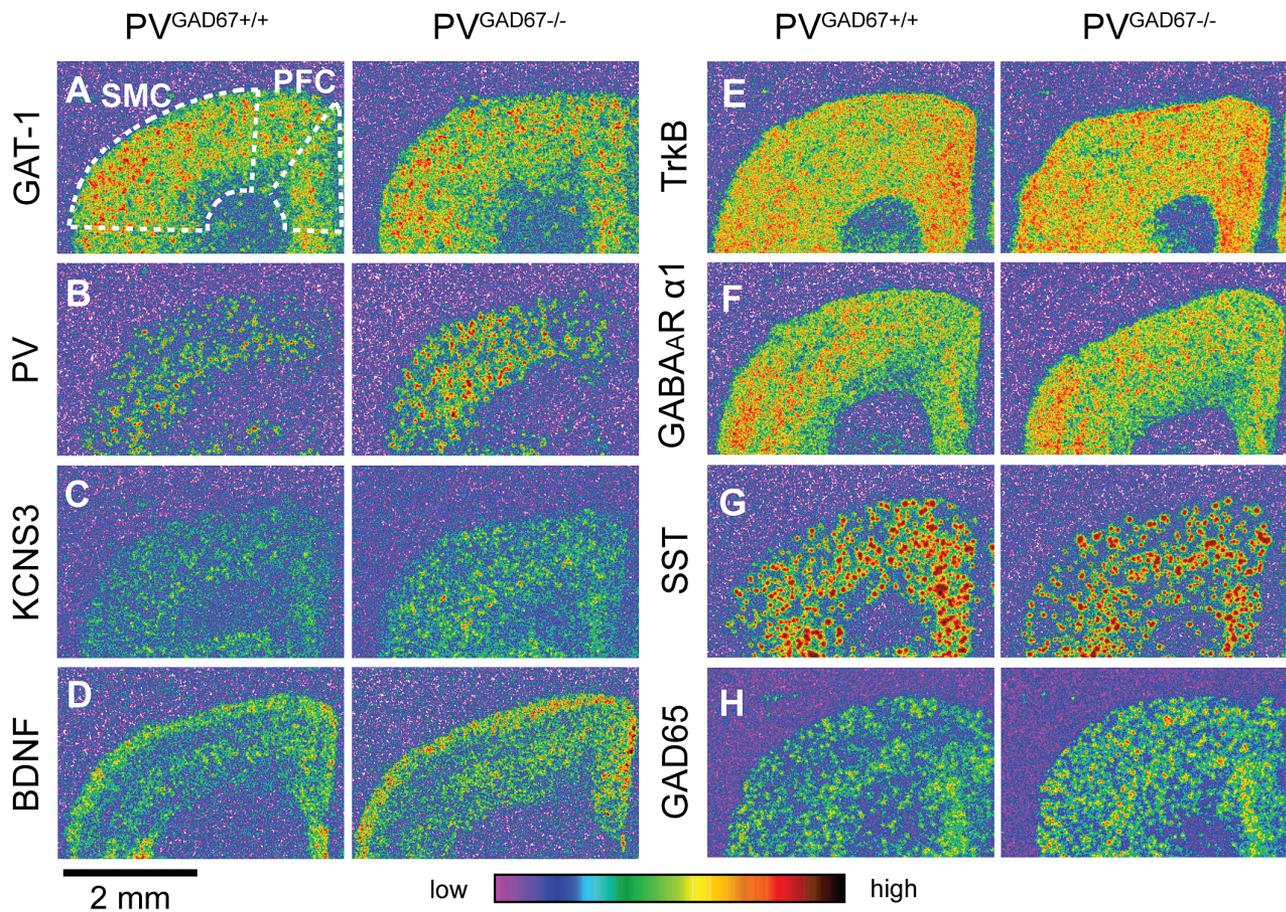
Fig. 2. Expression of GABA-associated messenger RNAs (mRNAs) in the cortex of mice with glutamic acid decarboxylase 67 (GAD67) knockout in parvalbumin (PV) neurons. Film images of the cortical expression of GABA transporter-1 (GAT-1; A), PV (B), KCNS3 (C), brain-derived neurotrophic factor (BDNF; D), tropomyosin receptor kinase B (TrkB; E), GABA$_A$R α1 (F), somatostatin (SST; G), and glutamic acid decarboxylase 65 (GAD65; H) mRNAs in PV$^{GAD67+/+}$ and PV$^{GAD67-/-}$ mice from the same triad at 8 weeks of age. The optical densities are presented in a pseudo-color manner according to the color bar at the bottom. Regions of interests in the prefrontal cortex (PFC) and sensorimotor cortex (SMC) are indicated by broken lines (A).

total time spent in the center field ($F_{2,15} = 0.47$, $P = .633$) or total time in the light chamber ($F_{2,15} = 0.07$, $P = .932$), respectively (figures 4A and 4C). On the other hand, in the light-dark transition test, PV$^{GAD67-/-}$ mice exhibited significantly higher light-dark transition number than both PV$^{GAD67+/-}$ and PV$^{GAD67+/+}$ mice ($F_{2,15} = 6.12$, $P = .011$; figure 4D), indicating that the homozygous knockout is associated with higher spontaneous activity. PV neuron-specific GAD67 knockout mice did not exhibit deficits in either sensorimotor gating (PPI) or working memory (Y-maze), behavioral hallmarks of schizophrenia. The ANOVA models failed to detect a significant effect of genotype ($F_{2,15} = 0.23$, $P = .797$) or genotype × prepulse intensity interaction ($F_{2,15} = 0.15$, $P = .858$) on percent PPI (figure 4E), or a significant effect of genotype on alternation rate ($F_{2,15} = 0.29$, $P = .750$, figure 4F) in the Y-maze test. However, we observed significant genotype effects on the entry number ($F_{2,15} = 4.32$, $P = .033$) and total distance traveled ($F_{2,15} = 4.58$, $P = .028$) in the Y-maze test (figures 4G and 4H). These measures were higher in PV$^{GAD67-/-}$ mice than in both PV$^{GAD67+/-}$ and PV$^{GAD67+/+}$

mice, although statistical significances were detected only between PV$^{GAD67-/-}$ and PV$^{GAD67+/-}$ mice (figures 4G and 4H). These observations are consistent with higher spontaneous activity of PV$^{GAD67-/-}$ mice (figure 4D).

Discussion

In order to test a causal relationship between reduced GAD67 expression in cortical PV neurons and lower expression levels of transcripts associated with GABA neuron functions, both of which are found in the cortex of subjects with schizophrenia,[1–13,17] we created mice with a PV neuron-specific GAD67 knockout. Although multiple measures confirmed that the genetic manipulation produced the expected gene dose-related reductions of GAD67 in cortical PV neurons, we failed to detect the predicted lower cortical expression of GABA-associated transcripts or the expected disturbance in sensorimotor gating or working memory in our conditional knockout mice. These findings indicate that reduced GAD67 expression in PV neurons is not a sufficient upstream
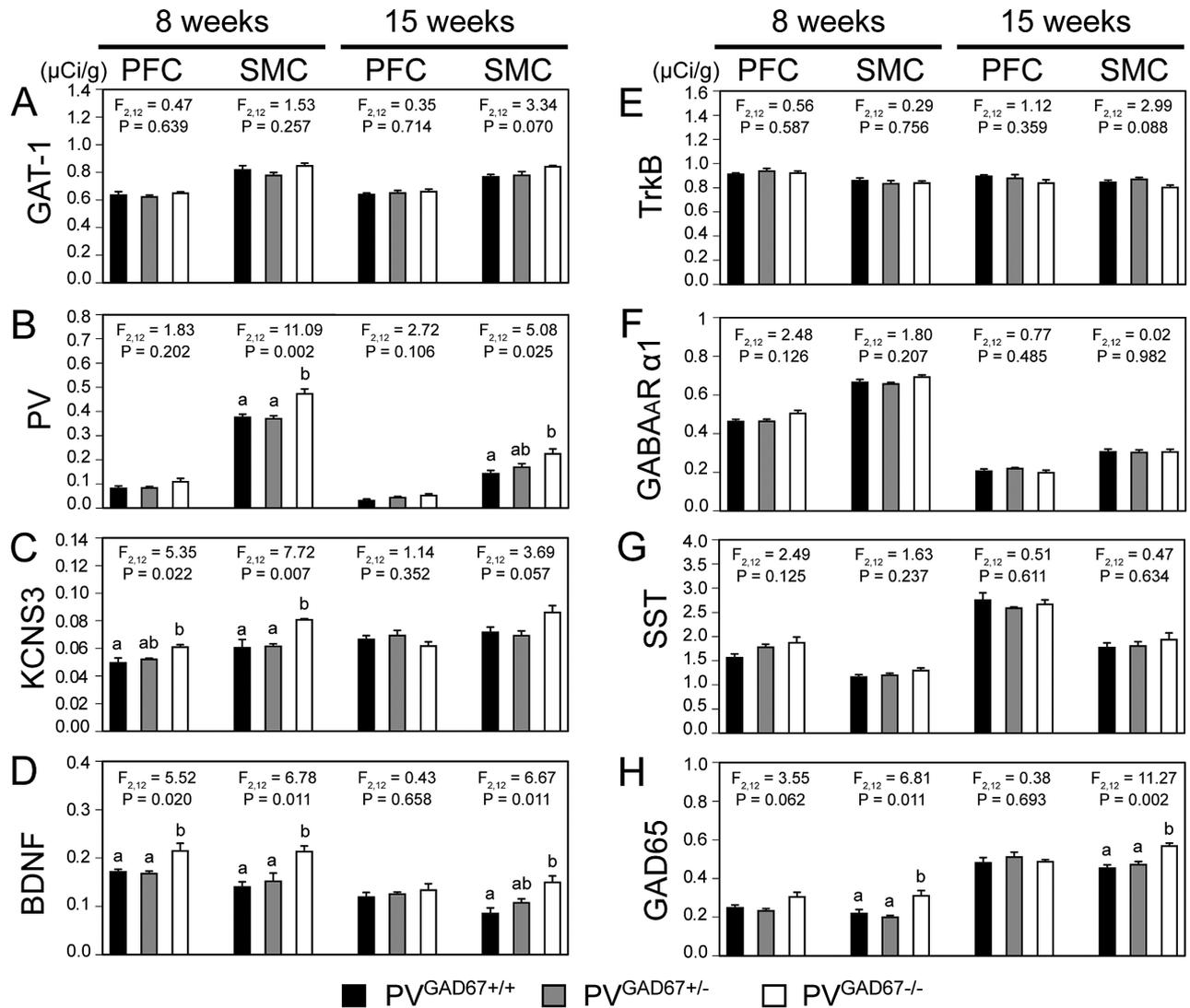
Fig. 3. Expression levels of GABA-associated mRNAs in the cortex of mice with glutamic acid decarboxylase 67 (GAD67) knockout in parvalbumin (PV) neurons. Mean ± SEM expression levels of GABA transporter-1 (GAT-1; A), PV (B), KCNS3 (C), brain-derived neurotrophic factor (BDNF; D), tropomyosin receptor kinase B (TrkB; E), GABA$_A$R α1 (F), somatostatin (SST; G), and glutamic acid decarboxylase 65 (GAD65; H) mRNAs in the prefrontal cortex (PFC) and sensorimotor cortex (SMC) of PV$^{GAD67+/+}$, PV$^{GAD67+/-}$, and PV$^{GAD67-/-}$ mice at 8 and 15 weeks of age. Bars not sharing the same letter are statistically different.

Table 1. Magnitude of Significant Increases in GABA-Associated Transcript Levels in the Homozygous (PV$^{GAD67-/-}$) Mice

mRNA	8 Weeks		15 Weeks	
	PFC	SMC	PFC	SMC
PV	ns	+25.7%	ns	+57.9%
KCNS3	+22.8%	+33.6%	ns	ns
BDNF	+25.3%	+52.4%	ns	+75.5%
GAD65	ns	+42.7%	ns	+25.3%

Note: mRNA, messenger RNA; PFC, prefrontal cortex; SMC, sensorimotor cortex; PV, parvalbumin; BDNF, brain-derived neurotrophic factor; GAD65, glutamic acid decarboxylase 65; ns, The effect of genotype on mRNA levels in ANOVA was not significant.

cause of the downregulated expression of other GABA-associated mRNAs or of the cognitive/behavioral impairments present in subjects with schizophrenia.

Reduced GAD67 and PV expression in cortical PV neurons was observed in animal models of *N*-methyl-D-aspartate glutamate receptor (NMDAR) hypofunction,[40,41] which has been suggested to underlie cortical dysfunction in schizophrenia.[42] In these models, the activity-dependent downregulation of GAD67[43] and PV[44] expression was the predicted consequence of reduced NMDAR signaling in PV neurons.[45] However, because NMDAR hypofunction in PV neurons would also be predicted to cause disinhibition of pyramidal neurons, as seen following systemic administration of NMDAR antagonists and genetic inactivation of NMDAR in PV
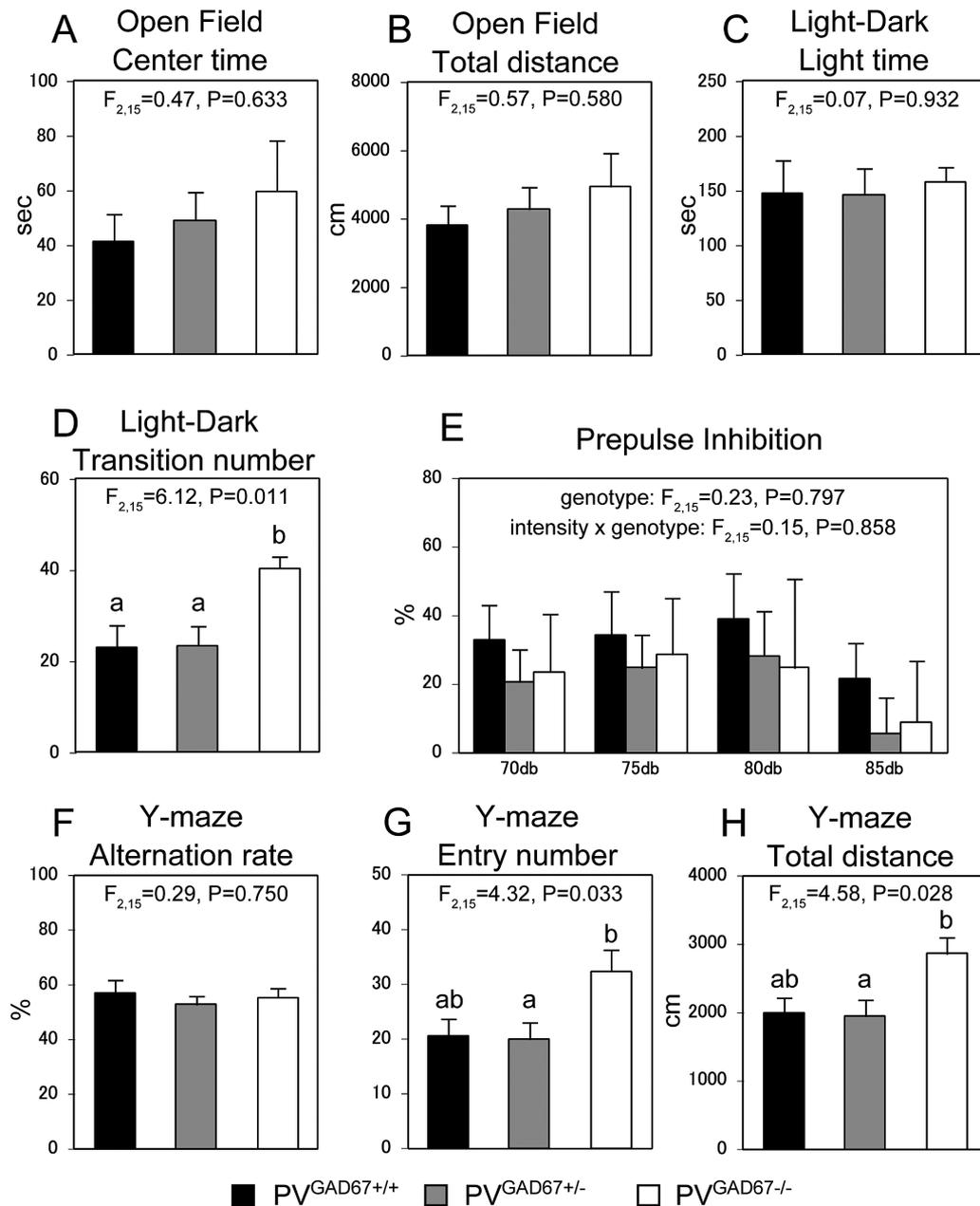
Fig. 4. Effects of parvalbumin (PV) neuron-specific glutamic acid decarboxylase 67 (GAD67) knockout on mouse behavior. Mean ± SEM behavioral measures of $PV^{GAD67+/+}$, $PV^{GAD67+/-}$, and $PV^{GAD67-/-}$ mice ($n = 6$ for each genotype) in the open filed (A and B), light–dark transition (C and D), prepulse inhibition (PPI; E), and Y-maze (F–H) tests. Bars not sharing the same letter are statistically different.

neurons,[41,45] other activity-dependent GABA-associated transcripts (ie, BDNF, TrkB and GABA$_A$R α1)[25–27] would also be expected to be upregulated in pyramidal neurons. However, these predictions are contrary to the lower levels of these transcripts observed in schizophrenia. Our current data also indicate that the GAD67 deficit in PV neurons does not cause reduced expression of other GABA-associated transcripts. Interestingly, the contribution of NMDAR to excitatory synaptic transmission and action potential generation is much larger in pyramidal neurons than in PV neurons.[46] Therefore, in schizophrenia, NMDAR hypofunction in pyramidal neurons could

cause activity-dependent downregulation of BDNF, TrkB, and GABA$_A$R α1 mRNAs. Such a state of pyramidal neuron NMDAR hypofunction could result from a deficit in pyramidal neuron dendritic spines, a major site of NMDAR localization, in schizophrenia.[47,48]

The following lines of information indicate that our analyses of the PV neuron-specific GAD67 knockout mice provided a robust means for proof-of-concept testing of the hypothesis that GAD67 deficits in PV neurons are responsible for lower GABA-associated transcript levels and behavioral abnormalities in schizophrenia. First, although our film-based quantification of

transcript levels might have limited resolution and sensitivity to detect alterations that are restricted to a minor subpopulation of neurons, most postmortem studies of schizophrenia to date have examined tissue-level expression of GABA-associated transcripts. Thus, we used these measures in order to maximize comparisons to the human disease. Second, we confirmed at the cellular level marked deficits in GAD67 expression in our PV[GAD67−/−] mice (figure 1) which parallel the reduction of GAD67 mRNA to undetectable levels in a subset of cortical GABA neurons, including PV neurons, in schizophrenia.[1,32,33] Third, we analyzed transcript levels at both 8 and 15 weeks of age and behaviors between 11 and 15 weeks; this age range corresponds to early adulthood in humans, a period when the diagnosis of schizophrenia is often made and characteristic behavioral and cognitive symptoms are evident. Thus, the age of mice examined in this study was appropriate for behavioral assessments. Fourth, although older mice might be better for testing GABA-associated transcript levels, as most postmortem studies of schizophrenia have used subjects with mean age greater than 40 years, cortical levels of KCNS3, BDNF, TrkB, and SST mRNAs, which showed negative correlations with age in both schizophrenia and control subjects, are lower in schizophrenia subjects compared with age-matched control subjects across a wide age range from 20 to 70 years of age.[7,9,17] These findings indicate that the levels of some GABA-associated transcripts are lower early in the course of illness.

Despite the appropriateness of our model for hypothesis testing, PV[GAD67−/−] mice exhibited increased cortical mRNA levels for PV, KCNS3, BDNF, and GAD65 (table 1) in contrast to the deficits in these transcripts present in schizophrenia. These findings provide compelling evidence against a primary problem in PV neurons as the cause of GABA-associated transcript alterations in schizophrenia. At 8 weeks, these increases were more prominent in the SMC than the PFC, especially for PV and GAD65 mRNAs that showed a significant increase only in the SMC, but not in the PFC. In the mouse PFC, unlike the human PFC, PV mRNA expression is lower than in other cortical areas (figure 2B),[7] reflecting the presence of fewer PV neurons.[49] Therefore, the smaller mRNA changes in the PFC relative to the SMC might reflect a lesser impact of the GAD67 knockout on PFC circuit. At 15 weeks, we observed significantly increased expression of PV, BDNF, and GAD65 mRNAs only in the SMC of PV[GAD67−/−] mice, and none of these transcripts showed a significant change in the PFC (table 1), suggesting that the impact of PV neuron-specific GAD67 knockout was effectively compensated in the PFC at 15 weeks of age.

In our PV-Cre mice, Cre activity becomes detectable in the cortex at P12.[50] Furthermore, in the previous study, the heterozygous GAD67 knockout in PV neurons resulted in a nonsignificant 9% decrease at P17 and a significant

18% decrease in GAD67 mRNA levels at P30.[38] Thus, our GAD67 knockout is likely to have started at the end of the second postnatal week, after the shift in GABA actions from depolarization to hyperpolarization.[51] In the mouse cortex, PV neurons strongly inhibit pyramidal neurons and PV neurons through perisomatic innervations with little inhibition to other GABA neuron subsets.[29] Thus, the primary effect of GAD67 knockout in PV neurons should be disinhibition of both pyramidal neurons and PV neurons. Neuronal activity regulates expression of BDNF mRNA in pyramidal neurons[26] and PV mRNA in PV neurons.[44] KCNS3 mRNA expression, which is selective to cortical PV neurons,[14,15,17] is also regulated by neuronal activity.[52] Therefore, increased levels of BDNF, PV, and KCNS3 mRNAs appear to reflect hyperactivity of both pyramidal and PV neurons. Consistent with this interpretation, a reduction of inhibitory postsynaptic current (IPSC) charge from PV neurons to pyramidal neurons and an increase in excitability of both pyramidal neurons and PV neurons were observed in the PFC of mice with a heterozygous GAD67 knockout in PV neurons from P23–P30.[50] Although this reduction in IPSCs was normalized by P50–P60 presumably through compensatory mechanisms,[50] increased BDNF, PV, and KCNS3 mRNA expression in the SMC of PV[GAD67−/−] mice suggest that the greater GAD67 deficit in our homozygous mice was not fully compensated in the SMC and resulted in hyperactivity of pyramidal neurons and PV neurons even at 8 and 15 weeks of age.

Two studies have analyzed cortical expression of some GABA-associated transcripts and proteins in independently generated mouse lines with a GAD67 knockout in PV neurons.[38,53] In one study, neither GAT-1 nor PV mRNA levels were significantly altered in the heterozygous mice compared with their wild type littermates,[38] consistent with our observation in PV[GAD67+/−] mice. In another study,[53] cortical GAD65 protein levels were unaltered in heterozygous mice and significantly increased in homozygous mice, corroborating our observation in the SMC of PV[GAD67+/−] and PV[GAD67−/−] mice. Increased GAD65 expression in homozygous mice might reflect a compensatory response to pyramidal neuron hyperactivity due to the extensive GAD67 deficit in PV neurons.

Studies of rodent models demonstrated that alterations of PFC GABA neurotransmission cause abnormalities of sensorimotor gating and working memory.[41,54,55] However, our homozygous PV[GAD67−/−] mice did not exhibit an abnormality in percent PPI or Y-maze alternation rate during 11–15 weeks of age (figures 4E and 4F), despite the significant GAD67 deficit in the PFC. These findings, together with the pattern of expression changes in GABA-associated transcripts in the PFC at 8 and 15 weeks (table 1), indicate that the impact of our PV neuron-specific GAD67 knockout on PFC GABA neurotransmission was effectively compensated after the maturation. Fujihara et al[53] reported impaired PPI in

mice with a GAD67 knockout primarily induced in PV neurons. This could be due to an involvement other types of PFC GABA neurons, as PV neurons accounted for only 70% of cortical Cre recombinase-positive GABA neurons in their bacterial artificial chromosome (BAC) transgenic mice.[53] In contrast, PV neurons corresponded to 97% of Cre recombinase-positive cortical neurons in our PV-Cre mice.[56]

A series of studies tested effects of GABA neuron subset-selective reduction of GAD67 expression using BAC transgenic mice that express a synthetic microRNA under the control of GABA neuron subset-specific promoters.[57–60] These studies revealed that GAD67 deficits in different subsets of GABA neurons resulted in distinctive, yet partially overlapping, behavioral phenotypes. Recently, a deficit in PPI was reported in such BAC transgenic mice with reduced GAD67 expression in PV neurons.[60] The discrepancy with unaltered PPI in our knockout mice might be due to differences in the cell-type specificity and timing of GAD67 reduction in the BAC-driven microRNA silencing of GAD67 mRNA vs the excision of the floxed *Gad1* gene by Cre expressed from the *Pvalb* gene locus. Nonetheless, as a direct demonstration of altered GABA neurotransmission is not available in our PV neuron-specific GAD67 knockout mice, we cannot exclude the possibility that the absence of a PPI change could be due to an insufficient functional impact of our genetic manipulation on GABA neurotransmission by PV neurons. However, this scenario appears unlikely, especially in our homozygous mice, because we observed upregulations of BDNF, PV, KCNS3, and GAD65 mRNAs, which are associated with enhanced neuronal activity and a compensation in response to reduced GABA neurotransmission by PV neurons.

Given that the findings of the present study failed to support the hypothesis that reduced GAD67 expression in PV neurons is the upstream cause of other GABA-associated transcript alterations in schizophrenia, what other mechanisms might explain these abnormalities? In schizophrenia, excitatory inputs to pyramidal neurons appear to be reduced, as indicated by a reduced density of dendritic spines.[47,48] This could cause pyramidal neuron hypoactivity and an activity-dependent downregulation of BDNF,[26] TrkB,[27] and GABA$_A$R α1 subunit[25] expression in these neurons. Furthermore, resultant weaker excitatory drive from pyramidal neurons to GABA neurons could drive down GAD67,[43] PV,[44] KCNS3,[52] and SST[61] mRNA levels. Therefore, the lower expression levels of GAD67 and other GABA-associated transcripts might be a consequence of pyramidal neuron hypoactivity in schizophrenia.[62]

Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

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