

Glutamate Decarboxylase 67 Deficiency in a Subset of GABAergic Neurons Induces Schizophrenia-Related Phenotypes

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Decreased expression of the GABA synthetic enzyme glutamate decarboxylase 67 (GAD67) in a subset of GABAergic neurons, including parvalbumin (PV)-expressing neurons, has been observed in postmortem brain studies of schizophrenics and in animal models of schizophrenia. However, it is unclear whether and how the perturbations of GAD67-mediated GABA synthesis and signaling contribute to the pathogenesis of schizophrenia. To address this issue, we generated the mice lacking GAD67 primarily in PV neurons and characterized them with focus on schizophrenia-related parameters. We found that heterozygous mutant mice exhibited schizophrenia-related behavioral abnormalities such as deficits in prepulse inhibition, MK-801 sensitivity, and social memory. Furthermore, we observed reduced inhibitory synaptic transmission, altered properties of NMDA receptor-mediated synaptic responses in pyramidal neurons, and increased spine density in hippocampal CA1 apical dendrites, suggesting a possible link between GAD67 deficiency and disturbed glutamatergic excitatory synaptic functions in schizophrenia. Thus, our results indicate that the mice heterozygous for GAD67 deficiency primarily in PV neurons share several neurochemical and behavioral abnormalities with schizophrenia, offering a novel tool for addressing the underlying pathophysiology of schizophrenia.

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INTRODUCTION

Schizophrenia is a major psychiatric disorder and is characterized by three classes of signs and clinical symptoms: positive symptoms (auditory hallucinations, delusions, and disorganized speech); negative symptoms (blunted affect and social withdrawal); and cognitive dysfunctions (Braff *et al*, 2001; Couture *et al*, 2006; Piskulic *et al*, 2007). Recent studies have revealed that schizophrenia develops through complex interactions between multiple genes and environmental risk factors (Insel, 2010). However, because of the etiological heterogeneity of schizophrenia, the molecular and cellular mechanisms in schizophrenia pathogenesis remain largely unknown. Thus, it is important to identify a common pathway that contributes to schizophrenia pathogenesis.

One of the most consistent findings in postmortem brain studies of schizophrenics is the decreased expression of the 67-kDa isoform of glutamate decarboxylase (GAD67), the primary GABA-synthesizing enzyme, in a subpopulation of GABAergic neurons (Guidotti *et al*, 2000; Hashimoto *et al*, 2003, 2008; Volk *et al*, 2000) including parvalbumin (PV)-expressing cells (Hashimoto *et al*, 2003) and somatostatin (SST)-expressing cells (Hashimoto *et al*, 2008) in the cerebral cortex and hippocampus. Similar GAD67 reductions have been observed in different animal schizophrenia models including NMDA receptor hypo-functioning in mice and the dopamine sensitization model in rats (Behrens *et al*, 2007; Lee *et al*, 2013; Liu *et al*, 2001; Takao *et al*, 2013). These studies suggest that GABAergic neuron dysfunction, primarily in PV neurons, is involved in schizophrenia pathophysiology, and this dysfunction may be a common process of the disease even though the etiology is heterogeneous. However, it is unknown whether the alteration is a cause of schizophrenia-related symptoms or merely an effect of the disease process.

Here, we report the generation and analyses of a conditional GAD67-knockout mouse strain in which GAD67 is

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deleted in a subset of GABAergic neurons, primarily in PV neurons, to address the cause and effect relationship between GAD67 deficiency and schizophrenia pathogenesis. We found that haploinsufficiency of the GAD67 gene in a subset of GABAergic neurons induced schizophrenia-related behavioral phenotypes and synaptic dysfunction in the hippocampus. Therefore, our results support the hypothesis that GAD67 deficiency is a possible common pathway that contributes to schizophrenia pathogenesis.

MATERIALS AND METHODS

A detailed description of the Materials and Methods is provided in the Supplementary Information.

Animals

PV-Cre; GAD67^{lox/lox} and *PV-Cre; GAD67^{lox/+}* mice were generated by crossing *Pvalb-Cre* BAC transgenic (Tg(*Pvalb-cre*); hereafter, *PV-Cre*) mice (Tanahira et al, 2009) and *GAD67-floxed (Gad1^{Tm1})* mice (Obata et al, 2008). In behavioral tests and acute slice electrophysiology, male *PV-Cre; GAD67^{lox/+}* mutant mice and littermate male controls (*GAD67^{lox/+}*) were used. All experiments were performed in accordance with the guidelines of the Animal Care and Experimentation Committee of the Gunma University and the Animal Research Committee of the Kumamoto University. All efforts were made to minimize the number of animals used and their suffering.

Western Blot Analysis

A standard Western blotting procedure was performed as described in the Supplementary Information, using anti-GAD65/67 (1 µg/ml) and anti-β-actin antibody (1 : 10 000, Abcam). Protein levels were quantified by densitometry of the immunochemical signal using ImageJ software and normalized to the signal intensity obtained for β-actin.

Immunohistochemistry

Perfusion and immunohistochemistry were performed as described in the Supplementary Information, using rabbit anti-PV antibody (1 : 5000, Swant), mouse anti-PV (1 : 5000, Swant), monoclonal mouse anti-GAD67 antibody (1 : 1000, Millipore), rabbit anti-GABA (1 : 1000, Sigma), rabbit anti-calretinin (1 : 2000, Swant), and rabbit anti-SST-14 (1 : 2000, Peninsula Laboratories). To visualize perineuronal nets (PNN), brain sections were incubated with the biotinylated lectin *Wisteria floribunda* agglutinin (1 : 2000, Vector Labs) at 4 °C overnight. After washing in PBS, the sections were incubated with Alexa Fluor 594 conjugated to streptavidin for 20 min at room temperature. For quantitative analysis, images were captured with a confocal laser scanning microscope (FV1000, Olympus) for GAD67, PV, calretinin, and SST or with a conventional epifluorescence microscope for GABA and PV double staining. The images were analyzed using ImageJ software. For each brain slice, all PV-, GAD67-, or GABA-positive cells were visually identified, and the intensities of the immunoreactive signals from the cell somata of each slice were measured. For GABA and PV double immunohistochemistry, the signal intensities

were measured and then transformed into Z-scores using the mean and SD of the control mice to equalize the variability of the immunohistochemical staining among the different samples due to differences in anti-GABA antibody penetration. Quantification of the fluorescent intensities was carried out without any adjustment of brightness/contrast. Stereological cell counting approach was employed from rectangular regions-of-interests (ROIs) of fixed size positioned over the prefrontal cortex (1000-µm high × 500-µm wide). Fluorescent cells within each ROI were counted using the Cell Counter plugin for ImageJ.

Slice Preparation and Electrophysiology

Hippocampal slices were prepared from 12- to 16-week-old male *PV-Cre; GAD67^{lox/+}* mice and littermate controls (*GAD67^{lox/+}*), using standard procedures (Miwa et al, 2008). Slices were perfused with a medium that was saturated with 95% O₂ and 5% CO₂ and contained 119 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.0 mM NaH₂PO₄, 26.2 mM NaHCO₃, and 11 mM glucose. The medium (at 25 ± 1 °C) for recordings containing 100 µM picrotoxin, a GABA_A receptor antagonist, was used for all of the experiments except for the recording of GABA_A receptor-mediated inhibitory postsynaptic currents (GABA_A-IPSCs). Whole-cell patch-clamp recordings were made from pyramidal cells in the CA1 region with a MultiClamp 700B patch-clamp amplifier (Molecular Devices, Union City, CA, USA). The pipette solution contained 122.5 mM cesium gluconate, 17.5 mM CsCl, 10 mM HEPES, 0.2 mM EGTA, 8 mM NaCl, 2 mM Mg-ATP, and 0.3 mM Na₃-GTP (pH 7.2; 290–310 mOsm). In some experiments, biocytin (5 mg/ml) was added to the internal solution immediately before recording. The recording electrodes had resistances of 3–7 MΩ. The series resistance was 10–30 MΩ and was monitored online throughout the experiment. The experiments were rejected if the series resistance changed by >20%. The signal was filtered at 5 kHz and digitized at 20 kHz with pClamp9.2 software (Molecular Devices). For evoking synaptic responses, a bipolar stimulating electrode was placed in the stratum radiatum and was stimulated at 0.1 Hz. The cells were voltage clamped at –90 mV to record excitatory postsynaptic currents (EPSCs), unless otherwise indicated. When NMDA receptor-mediated EPSCs were recorded, 10 µM CNQX was present to block AMPA receptor-mediated EPSCs (AMPA-EPSCs). GABA_A-IPSCs were evoked at 0 mV in the presence of CNQX (10 µM) and D-APV (50 µM). Experiments were carried out in a genotype-blinded manner.

Dendritic Spine Measurements

CA1 pyramidal neurons were filled with intracellular solution containing biocytin for at least 5–10 min, and then fixed. Biocytin was visualized with Alexa Fluor-594-streptavidin. Serial Z-stack images of secondary apical dendritic segments were obtained by confocal microscopy (Olympus FV1000 Fluoview confocal microscope; ×100 oil immersion objective (NA 1.4); 5.0 × zoom). The digitized stack images were deconvolved with AutoQuant X3 (Media Cybernetics, Rockville, MD, USA) and then analyzed using

Fiji (ImageJ) software. The number of spines was counted manually and spine density was calculated.

Behavioral Analyses

Open field test. Each mouse was placed in one corner of the apparatus (O'Hara, Tokyo, Japan) and was allowed to move freely for 30 min and then MK-801 (0.2 mg/kg; Sigma) was administered intraperitoneally, and the mouse was returned to the same arena for 90 min. Data were collected every 5 min and analyzed using ImageJ OFC software (O'Hara).

Acoustic startle response and PPI test. An acoustic startle reflex measurement system (O'Hara) was used. The startle response was assessed with various stimuli intensities. Five times of 70 to 120 dB (70, 75, 80, 85, 90, 95, 100, 110, and 120 dB) white noise stimuli (40 ms) were presented in quasi-random order and random inter-trial intervals (10–20 s). In the prepulse inhibition (PPI) session, mice experienced five trial types: no stimulus; startle stimulus (120 dB, 40 ms) only; prepulse 70 dB (20 ms, lead time 100 ms) and pulse 120 dB; prepulse 75 dB (20 ms, lead time 100 ms) and pulse 120 dB; and prepulse 80 dB (20 ms, lead time 100 ms) and pulse 120 dB. Each trial was repeated 10 times in quasi-random order and random inter-trial interval (10–20 s). PPI was defined as the percent decline of the startle response: $100 - [(startle\ amplitude\ after\ prepulse\ and\ pulse) / (startle\ amplitude\ after\ pulse\ only)] \times 100$.

Spontaneous alternation in the Y-maze. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8 min session. The percentage of spontaneous alternation was calculated as follows: the number of alternation (entries into three different arms consecutively) divided by the total possible alternations (ie, the number of arm entries – 2) $\times 100$.

Sociability and preference for social novelty test. The social test apparatus consists of a rectangular, three-chambered box illuminated by LEDs (O'Hara). A subject mouse was first placed in the middle chamber and habituated to the entire test box for 10 min. After habituation, the subject mouse was removed from the apparatus and an unfamiliar male mouse (stranger 1) that had no prior contact with the subject mouse was placed in one of the side chambers. The location of stranger 1 in the left or right side chamber was systematically alternated between trials. The stranger 1 mouse was enclosed in a small cylindrical wire cage. An identical empty cage was placed in the opposite chamber. The subject mouse was then placed in the middle chamber and was allowed to move freely for 10 min (sociability test). At the end of the sociability test session, the subject mouse was removed from the apparatus, and an additional novel unfamiliar mouse (stranger 2) was enclosed in the wire cage that had previously been empty. The subject mouse was placed in the middle chamber and allowed to explore the apparatus for additional 10 min to assess social memory (social novelty test). The mouse behavior was recorded and analyzed by ImageJ CSI software (O'Hara).

Statistics

Data are expressed as the mean \pm SEM, and were analyzed by Student's *t*-test, Holm's method after one-way ANOVA, or two-way repeated measures ANOVA unless otherwise stated. The log-rank test (Figure 1a and b), Dunnett's *post-hoc t*-test (Figure 2d), Mann–Whitney *U*-test (Figure 2e left), Welch's unpaired *t*-test (Figure 2e right), Bonferroni *post-hoc* test, or Shaffer's *post-hoc* test (Figure 4a–c) were also used. $P < 0.05$ were accepted as statistically significant.

RESULTS

GAD67 and GABA Levels Were Reduced in Mice Lacking GAD67 Primarily in PV-positive GABAergic Neurons

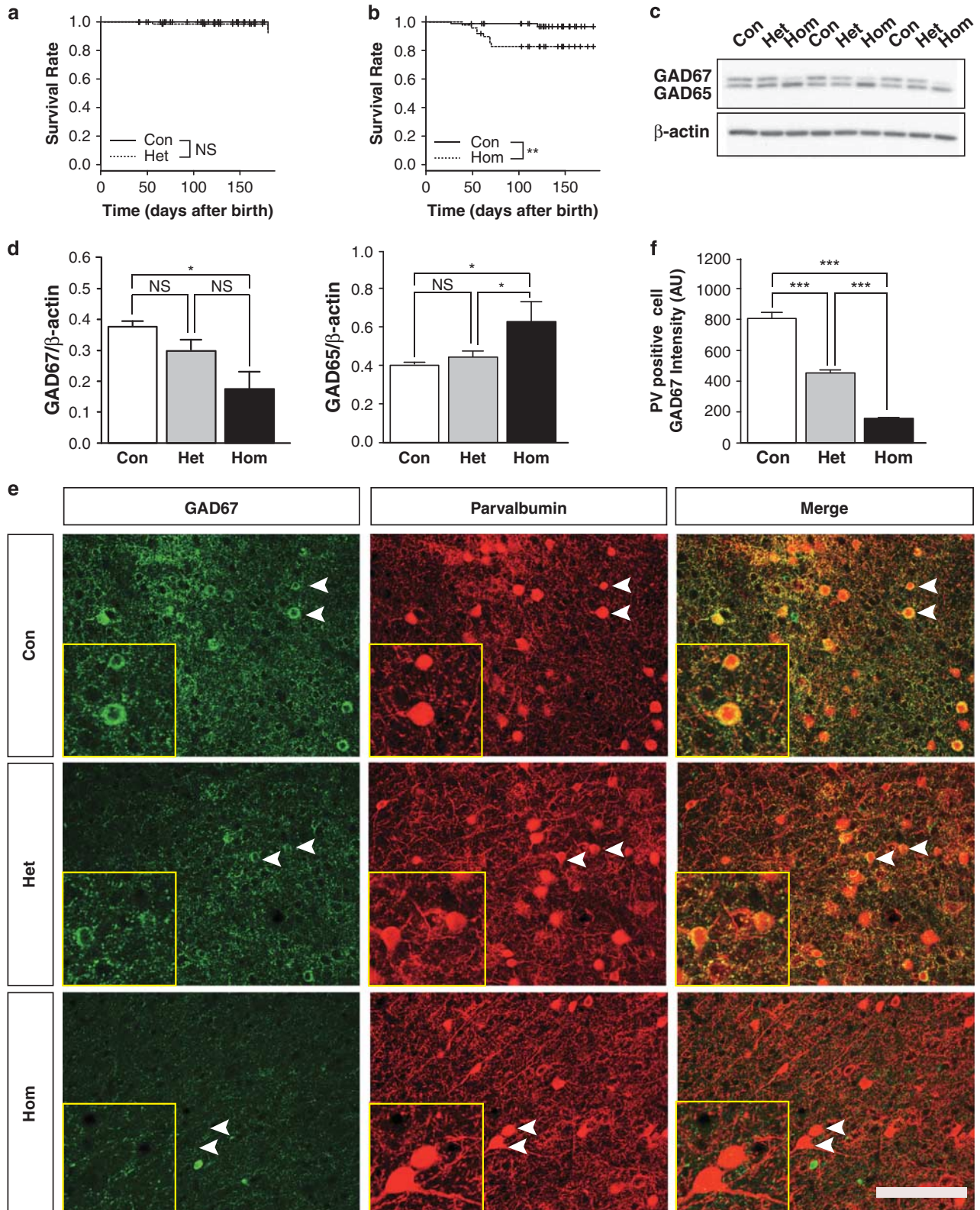
First, we confirmed the cell types targeted for Cre activity in the *PV-Cre* mouse by using a reporter mouse. As previously reported, Cre expression was detected primarily in PV-positive GABAergic neurons but also in certain other subtypes of GABAergic and pyramidal neurons (Tanahira *et al*, 2009). Because we focused on GABAergic neurons, we crossed the *PV-Cre* mouse with a *VGAT-floxed STOP-tdTomato* transgenic mouse. We observed that 95% of the PV-immunoreactive cells were positive for tdTomato in *PV-Cre*; *VGAT-floxed STOP-tdTomato* mice (data not shown). Approximately 70% of tdTomato-positive cells were positive for PV (Supplementary Figure S1 and Supplementary Table S1). In addition, tdTomato-positive but PV-negative cells were, at least in part, SST- or calretinin-positive GABAergic neurons (Supplementary Figure S1 and Supplementary Table S1). Thus, Cre activity in the *PV-Cre* mouse was detected in a subset of GABAergic neurons, primarily in PV neurons, which is consistent with a previous study (Tanahira *et al*, 2009).

Next, to test whether GAD67 deficiency in a subset of GABAergic neurons, primarily in PV neurons, leads to schizophrenia-like phenotypes, we generated *PV-Cre*; *GAD67^{flox/flox}* and *PV-Cre*; *GAD67^{flox/+}* mice (Supplementary Figure S2a and b) by crossing *GAD67-floxed* mice (Obata *et al*, 2008) with *PV-Cre* mice (Tanahira *et al*, 2009). Because PV is expressed in the ovary and testis (Kägi *et al*, 1987; Pohl *et al*, 1995), it is possible that undesired Cre-mediated recombination occurs in germ line (Kobayashi and Hensch, 2013). We observed *GAD67*-null mutant mice (ie, *GAD67^{flox/null}* and *PV-Cre*; *GAD67^{flox/null}*) at a frequency of $< 5\%$. We excluded these *GAD67*-null mutants from further analysis (Supplementary Figure S2c). Both homozygous *PV-Cre*; *GAD67^{flox/flox}* and heterozygous *PV-Cre*; *GAD67^{flox/+}* mice showed no gross abnormalities in their cytoarchitectures of the brain (data not shown). The survival rate of heterozygous *PV-Cre*; *GAD67^{flox/+}* mice was comparable to the control *GAD67^{flox/+}* mice (Con, $n = 116$; Het, $n = 70$; Figure 1a). However, the survival rate of homozygous *PV-Cre*; *GAD67^{flox/flox}* mice was lower than the control *GAD67^{flox/flox}* mice (Con, $n = 86$; Hom, $n = 49$; log-rank test, $P < 0.005$; Figure 1b) because certain homozygous *PV-Cre*; *GAD67^{flox/flox}* mice developed epileptic seizures with a significantly higher risk of seizure-related sudden death. Approximately 20% of these mice died by 2 months of age.

We quantified the protein amounts of two isoforms of glutamate decarboxylase (ie, GAD67 and GAD65) in the cerebral cortex through western blot analyses (Figure 1c).

GAD67 protein level was significantly reduced in *PV-Cre; GAD67^{lox/lox}* mice ($F(2, 6) = 6.43, P < 0.05$; one-way ANOVA followed by Holm's method, $P < 0.05$; Figure 1d left). GAD67 protein level in *PV-Cre; GAD67^{lox/+}* mice was also reduced but not significantly (Figure 1d left). In contrast, GAD65

protein level in *PV-Cre; GAD67^{lox/lox}* mice was significantly increased compared with the control mice and *PV-Cre; GAD67^{lox/+}* mice ($F(2, 6) = 10.64, P < 0.05$; one-way ANOVA followed by Holm's method, $P < 0.05$ Con vs Hom, $P < 0.05$ Het vs Hom $P < 0.05$; Figure 1d right). It should be



noted that the amounts of GAD67 and GAD65 in wild-type, $GAD67^{flox/+}$, and $GAD67^{flox/flox}$ mice were not significantly different (Supplementary Figure S3), which suggests that the altered GAD67 and GAD65 protein levels in $PV-Cre$; $GAD67^{flox/flox}$ mice can be attributed to Cre-mediated deletion of the GAD67 gene.

We further confirmed PV-Cre-mediated deletion of GAD67 through double immunohistochemistry for GAD67, GABA, and PV in the soma of PV-positive neurons. GAD67 immunoreactivity in the frontal cortex ($F(2, 106) = 168.3$, $P < 0.0001$; one-way ANOVA followed by Holm's method, $P < 0.0001$ Con vs Het, $P < 0.0001$ Con vs Hom; Figure 1e and f) and the hippocampal CA1 region (data not shown) was reduced in the mutant mice, reflecting the gene dosage, to nearly undetectable levels in $PV-Cre$; $GAD67^{flox/flox}$ mice. To evaluate the functional effects of the GAD67 reduction, we next examined GABA immunoreactivity (Figure 2). GABA immunoreactivity was significantly reduced in the mutant mice ($F(2, 1829) = 273.28$, $P < 0.0001$; one-way ANOVA followed by Tukey's method, $P < 0.0001$ Con vs Het, $P < 0.0001$ Con vs Hom; Figure 2b upper). Surprisingly, however, PV immunoreactivity was significantly increased in $PV-Cre$; $GAD67^{flox/flox}$ mice but not in $PV-Cre$; $GAD67^{flox/+}$ mice ($F(2, 1829) = 68.32$, $P < 0.0001$; one-way ANOVA followed by Tukey's method, $P < 0.0001$ Con vs Hom; Figure 2b bottom). Taken together, the GAD67 and GABA immunoreactivities in PV neurons of the mutant mice were reduced in a gene dose-dependent manner, whereas the GAD65 and PV immunoreactivities were increased in $PV-Cre$; $GAD67^{flox/flox}$ mice. To further evaluate the impact of GAD67 decrease in PV neurons on PV neuron dysfunction, we quantified the proportion of PV cell enwrapped with PNNs, specialized structures of the neural extracellular matrix (Kosaka and Heizmann, 1989; Figure 2c–e). The density of PNN-positive cells in $PV-Cre$; $GAD67^{flox/flox}$ mice was significantly increased, compared with the control mice ($F(2, 32) = 6.08$, $P < 0.01$; one-way ANOVA followed by Dunnett's t test, $P < 0.05$; Figure 2d). Therefore, these results suggest that homozygous GAD67 deletion in PV neurons leads to substantial increases in PV and GAD65 immunoreactivity and marked increase in PNNs as well as attenuated GABA levels and increases the risk of sudden, unexpected death due to epilepsy. However, because GAD67 expression in schizophrenia patients is not completely lost but is moderately decreased in PV neurons, and the primary aim of this study was to mimic the pathological changes of schizophrenia as far as possible (Guidotti *et al*, 2000; Todtenkopf

and Benes, 1998; Woo *et al*, 1997; Zhang and Reynolds, 2002), we used heterozygous $PV-Cre$; $GAD67^{flox/+}$ mice for further experiments. We found that the number of PV-positive cells ($P < 0.05$; Figure 2e left) and the number of PV cells with PNNs ($t(15.15) = 2.156$, $P < 0.05$; Figure 2e right) in $PV-Cre$; $GAD67^{flox/+}$ mice were significantly decreased, compared with the control mice, consistent with the post-mortem studies of subjects with schizophrenia (Mauney *et al*, 2013; Todtenkopf and Benes, 1998; Zhang and Reynolds, 2002).

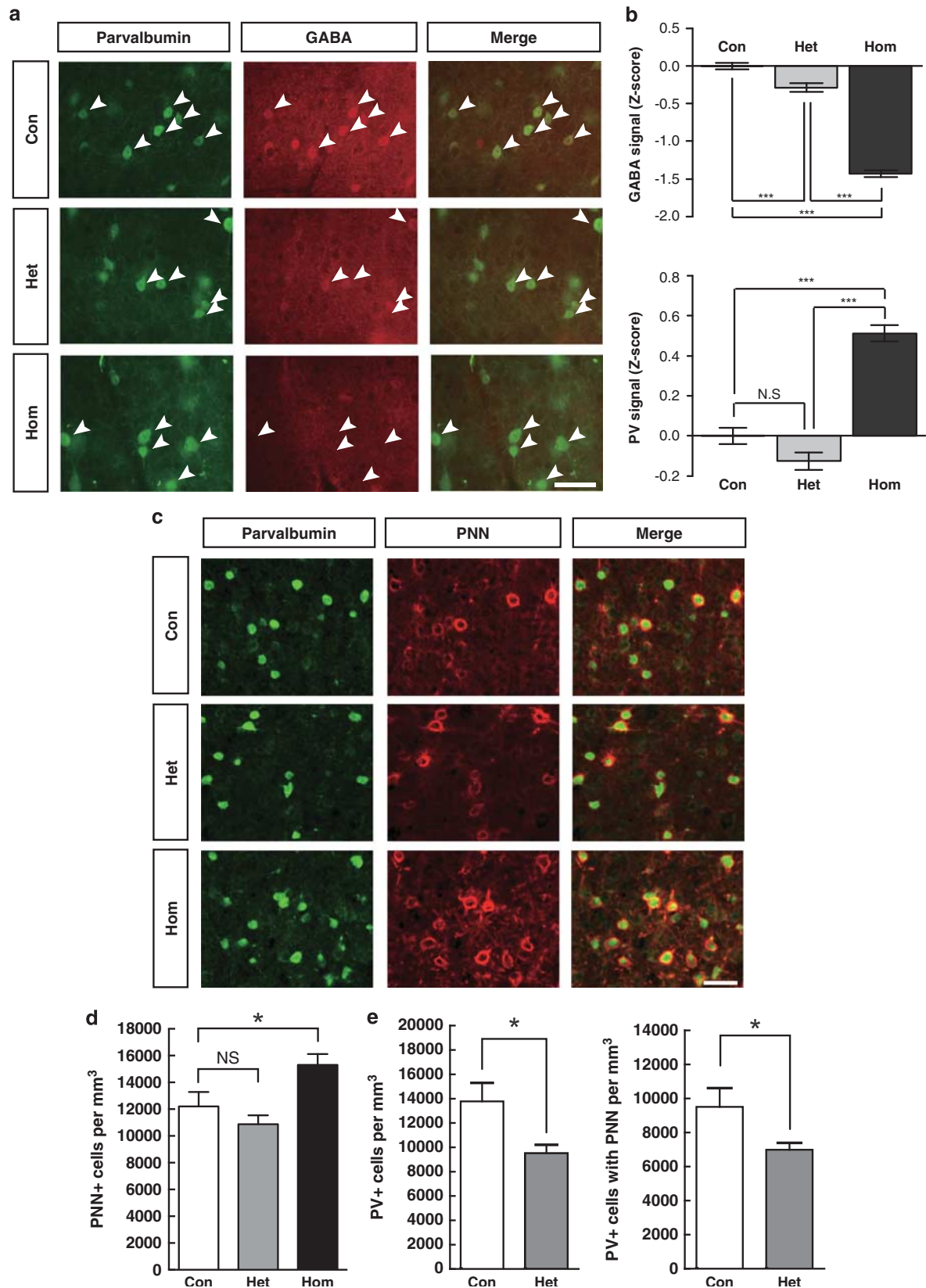
Synaptic Dysfunction in $PV-Cre$; $GAD67^{flox/+}$ Mice

We investigated synaptic function using whole-cell patch-clamp recordings in the CA1 region of the hippocampus, which displays progressive functional, structural, and neurochemical alterations in imaging and postmortem brain studies of patients with schizophrenia, as well as the neocortex (Todtenkopf and Benes, 1998; Zhang and Reynolds, 2002). To this end, synaptic responses were evoked by a bipolar stimulating electrode placed in the stratum radiatum of acute hippocampal slices. We first evaluated the effects of GAD67 deficiency in PV neurons on GABAergic, inhibitory synaptic transmission to pyramidal neurons. The input–output relationship of GABA_A-IPSCs was smaller in heterozygous $PV-Cre$; $GAD67^{flox/+}$ mice (two-way ANOVA, $F(1, 120) = 13.88$, $P < 0.0005$ for genotype; Figure 3a and b), which indicates that decreased inhibitory synaptic strength in $PV-Cre$; $GAD67^{flox/+}$ mice is attributable to decreased GAD67 expression in the PV neurons of $PV-Cre$; $GAD67^{flox/+}$ mice. Next, we investigated excitatory synaptic transmission because GABA regulates excitatory synapse formation (Ben-Ari *et al*, 2004). The input–output relationship of AMPA-EPSCs (Figure 3c and d) and the ratio of NMDA to AMPA EPSC amplitude (Figure 3e and f) were similar in both groups, suggesting that basal excitatory synaptic transmission remains intact in $PV-Cre$; $GAD67^{flox/+}$ mice. Interestingly, however, the current–voltage (I – V) curve of NMDA-EPSCs in $PV-Cre$; $GAD67^{flox/+}$ mice was lower than control mice (two-way ANOVA, $F(1, 15) = 11.52$, $P < 0.005$ for genotype; Figure 3g and h), and the membrane potential at which the NMDA receptor channel exhibited 50% of the maximal conductance (V_{50}) in conductance–voltage plots was higher (Supplementary Figure S4). The rise time and the fast (τ_f) and slow (τ_s) decay time constant of the NMDA-EPSCs (measured at +40 mV) were similar between the genotypes. To further examine synaptic dysfunction, we counted dendritic spines on biocytin-filled pyramidal neurons. We observed an increased

Figure 1 Mortality and GAD67 reduction in the cerebral cortex of heterozygous $PV-Cre$; $GAD67^{flox/+}$ and homozygous $PV-Cre$; $GAD67^{flox/flox}$ mice. (a and b) The survival rate of heterozygous $PV-Cre$; $GAD67^{flox/+}$ mice was comparable to the control mice, but the survival rate of homozygous $PV-Cre$; $GAD67^{flox/flox}$ mice was reduced. (a) Survival curves of $GAD67^{flox/+}$ (Con, $n = 116$) and $PV-Cre$; $GAD67^{flox/+}$ (Het, $n = 70$) mice (log-rank test, $P = 0.42$). (b) Survival curves of $GAD67^{flox/flox}$ (Con, $n = 86$) and $PV-Cre$; $GAD67^{flox/flox}$ (Hom, $n = 49$) mice (log-rank test, $P < 0.005$). *** $P < 0.01$. (c and d) Decreased GAD67 but increased GAD65 protein levels in homozygous $PV-Cre$; $GAD67^{flox/flox}$ mice. (c) Western blot analysis of the cerebral cortex of control $GAD67^{flox/+}$, heterozygous $PV-Cre$; $GAD67^{flox/+}$, and homozygous $PV-Cre$; $GAD67^{flox/flox}$ mice using anti-GAD65/67 antibody with β -actin as an internal control. (d) Densitometric analysis of the expression levels of GAD67 (left) and GAD65 (right). To evaluate the protein levels of GAD65 and GAD67, the intensities of their bands in c were divided by their corresponding control (β -actin). The data are presented as the mean \pm SEM. P -values were calculated using one-way repeated measures ANOVA followed by Holm's method. * $P < 0.05$. (e and f) Reduction of GAD67 immunoreactivity in PV-positive neurons of the frontal cortex. Controls (Con); heterozygous $PV-Cre$; $GAD67^{flox/+}$ (Het); homozygous $PV-Cre$; $GAD67^{flox/flox}$ mice (Hom). The results in e were quantified in f. $n = 9$ slices from three mice for each genotype (Con, Het, and Hom). *** $P < 0.001$; one-way repeated measures ANOVA. Scale bar, 100 μ m. ANOVA, analysis of variance; GAD67, glutamate decarboxylase 67; NS, not significant; PV, parvalbumin.

spine density in *PV-Cre; GAD67^{flox/+}* mice ($t(23)=2.562$, $P<0.05$; Figure 3i and j), compared with control mice. These data indicate that not only inhibitory synaptic function but also excitatory synaptic function are impaired in *PV-Cre;*

GAD67^{flox/+} mice, which suggests that heterozygous GAD67 deletion in a subset of GABAergic neurons, mainly in PV neurons, is sufficient to cause an imbalance of excitation and inhibition in the hippocampus.



Schizophrenia-Like Behavioral Features of *PV-Cre; GAD67^{fllox/+}* Mice

To determine whether the neurochemical and physiological disturbances in *PV-Cre; GAD67^{fllox/+}* mice produce behavioral impairments, we performed behavioral tests to assess schizophrenia-like traits. *PV-Cre; GAD67^{fllox/+}* mice appeared healthy and showed no obvious differences in neurological and physical characteristics, compared with the control mice (data not shown). We first tested the mice for global behavioral changes and detected no changes in anxiety-related behaviors and motor coordination/motor learning in *PV-Cre; GAD67^{fllox/+}* mice with a series of tests (light–dark transition test, elevated plus maze, Porsolt forced swim test, and rotarod test (Supplementary Figures 5)).

Hyperlocomotor activity has been correlated with the positive symptoms of schizophrenia (Lahti *et al*, 1995). In addition, treatment with noncompetitive NMDA receptor antagonists including ketamine, phencyclidine, and MK-801 causes schizophrenia-like psychosis in humans (Bubeníková-Valesová *et al*, 2008). Therefore, we exposed control mice and *PV-Cre; GAD67^{fllox/+}* mice to an open field for 30 min and then administered the psychostimulant MK-801 followed by continued exposure to the open field for 90 min. *PV-Cre; GAD67^{fllox/+}* mice showed normal spontaneous locomotion compared with control *GAD67^{fllox/+}* mice (1–30 min; Figure 4a). The rearing number of the mutant mice tended to be slightly higher than controls, but the difference was not significant (Figure 4b). The total center time was not significantly different between the two genotypes (Figure 4c). These results indicating that *PV-Cre; GAD67^{fllox/+}* mice have normal anxiety levels, and their open field locomotion is not affected by anxiety. After MK-801 administration, both control and *PV-Cre; GAD67^{fllox/+}* mice showed a progressive increase in locomotor activity, and MK-801-induced hyperlocomotion was significantly enhanced in *PV-Cre; GAD67^{fllox/+}* mice (31–120 min, $F(1, 14) = 11.07$, $P < 0.005$ for genotype; Figure 4a). The rearing number and center time of *PV-Cre; GAD67^{fllox/+}* tended to be slightly higher than controls after MK-801 administration but not to a significant degree (Figure 4b and c). Thus, these data suggest that *PV-Cre; GAD67^{fllox/+}* mice show more sensitivity to the locomotor-stimulating effects of MK-801.

PPI of the startle reflex, a measure of sensorimotor gating, is a well-characterized endophenotype in schizophrenia. PPI deficits have been repeatedly found in schizophrenia patients (Braff *et al*, 2001). We observed impaired PPI of the acoustic startle response at prepulse intensities of 70 and 75 dB in *PV-Cre; GAD67^{fllox/+}* mice ($F(1, 75) = 21.02$, $P < 0.005$ for

genotype effect; Two-way repeated measures ANOVA followed by Holm's *post-hoc* test, $P < 0.05$ for 70 dB, $P < 0.05$ for 75 dB, and $P = 0.31$ for 80 dB; Figure 4d), whereas the amplitudes of the acoustic startle response were similar between the two genotypes (Figure 4e), thereby excluding sensorimotor defects.

Cognitive dysfunction is considered a clinical feature of schizophrenia (Piskulic *et al*, 2007). To examine cognitive function in *PV-Cre; GAD67^{fllox/+}* mice, we used the Y-maze spontaneous alternation task (Paul *et al*, 2009). No difference was observed in the spontaneous alternation rates (Figure 4f). In addition, contextual and cued fear conditioning tests were conducted to examine learning and memory. No genotypic effects in fear conditioning or in contextual and cued fear memories were observed between the two genotypes (Supplementary Figure S9).

Social behavioral deficits including social withdrawal and isolation are frequently observed in schizophrenia patients and are key components of the negative symptoms of schizophrenia (Couture *et al*, 2006). To this end, a three-chamber social interaction test was performed (Nadler *et al*, 2004). In a sociability test for social vs empty preference, both the control and *PV-Cre; GAD67^{fllox/+}* mice spent significantly more time interacting with the stranger than with the empty cage (paired *t*-test, Con: $t(17) = 4.232$, $P < 0.001$, Het: $t(14) = 4.750$, $P < 0.0005$, respectively; Figure 4g), which indicates that the sociability of *PV-Cre; GAD67^{fllox/+}* mice remained intact. Subsequently, we examined a preference for social novelty, which relies on social memory. Control mice spent more time interacting with the novel mouse than with the familiar mouse. In contrast, *PV-Cre; GAD67^{fllox/+}* mice did not show a preference for social novelty (paired *t*-test, Con: $t(17) = -2.365$, $P < 0.05$; $t(14) = -0.319$, Het: $P = 0.755$; Figure 4h). These results indicate that *PV-Cre; GAD67^{fllox/+}* mice have normal sociability but showed an impaired social novelty preference (ie, deficits in social memory). Collectively, our results suggest that *PV-Cre; GAD67^{fllox/+}* mice exhibit schizophrenia-like behaviors.

DISCUSSION

One of the most highly replicated findings in postmortem brain studies of subjects with schizophrenia is decreased GAD67 expression in a subset of GABAergic neurons, including PV neurons. However, it has been unclear whether and how the alteration is a cause of schizophrenia-related endophenotypes or merely an effect of the disease process. We found that *PV-Cre; GAD67^{fllox/+}* mice show multiple

Figure 2 Immunohistochemical characterization of PV neuron dysfunction in the cerebral cortex of heterozygous *PV-Cre; GAD67^{fllox/+}* and homozygous *PV-Cre; GAD67^{fllox/fllox}* mice. (a) Double immunohistochemistry for PV (green) and GABA (red) in the frontal cortex of controls (Con), heterozygous *PV-Cre; GAD67^{fllox/+}* (Het), and homozygous *PV-Cre; GAD67^{fllox/fllox}* mice (Hom). Scale bar, 50 μ m. (b) Signal intensities obtained from each PV-positive somata were transformed into Z-scores (normalized by the average and the SD of the data set of control *GAD67^{fllox/+}* mice; upper, GABA; bottom, PV). $n = 9$ slices from three mice for each genotype (Con, Het, and Hom). $**P < 0.01$, $***P < 0.001$. The error bars indicate the mean \pm SEM one-way ANOVA followed by Holm's method. (c) Double immunohistochemistry for PV (green) and PNNs (red) in the frontal cortex of controls (Con), heterozygous *PV-Cre; GAD67^{fllox/+}* (Het), and homozygous *PV-Cre; GAD67^{fllox/fllox}* mice (Hom). Scale bar, 50 μ m. (d) Quantification of the number of PNN-positive cells. $n = 10$ –13 slices from three mice for each genotype (Con, Het, and Hom). $*P < 0.05$. The error bars indicate the mean \pm SEM one-way ANOVA followed by Dunnett's method. (e) Quantification of the number of PV-positive cells (left), and PV-neurons with PNNs (right). $n = 10$ –13 slices from three mice for each genotype (Con, Het). $*P < 0.05$; left, Mann–Whitney *U*-test; right, Welch's unpaired *t*-test. ANOVA, analysis of variance; GAD67, glutamate decarboxylase 67; NS, not significant; PV, parvalbumin.

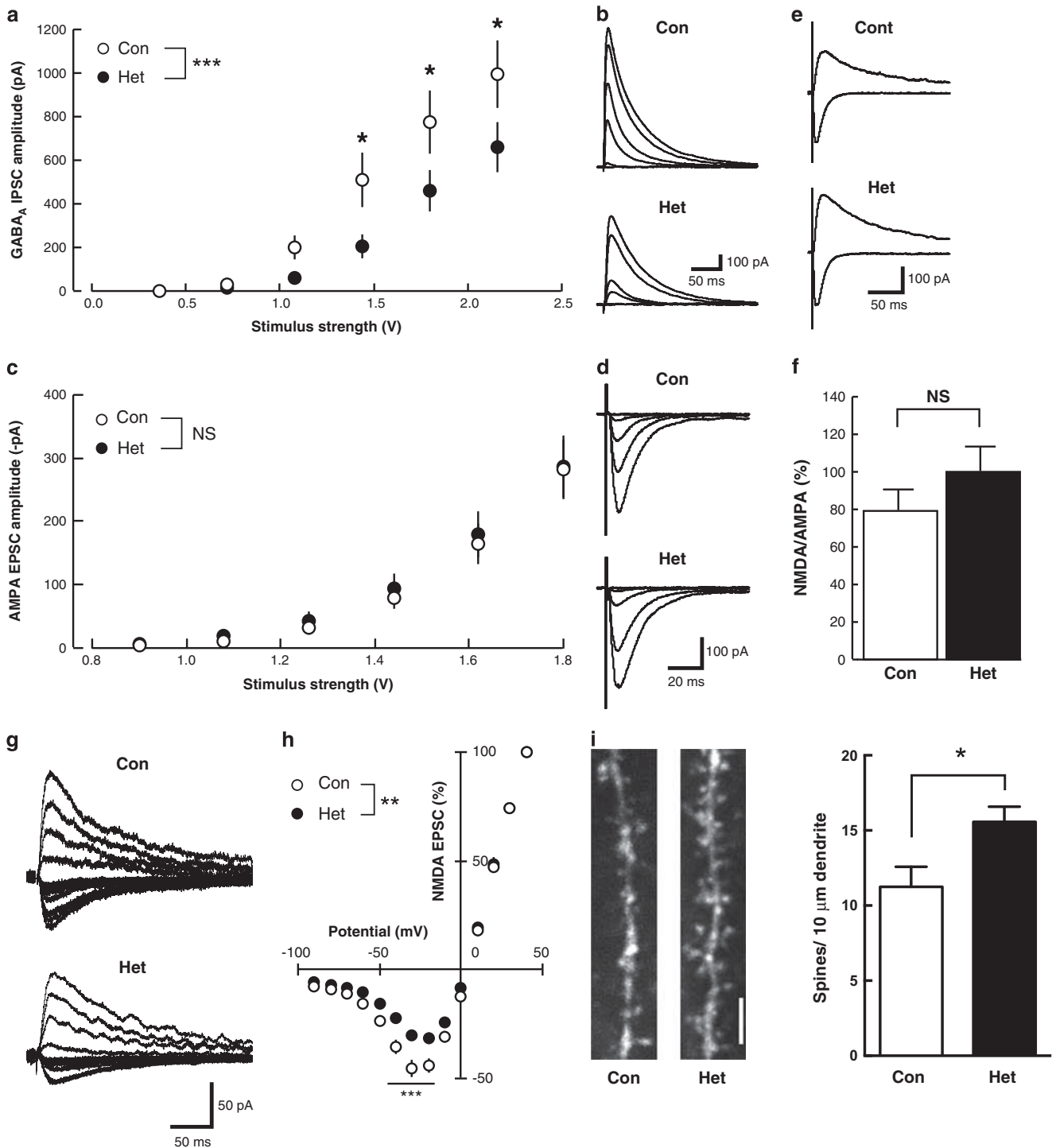


Figure 3 Disrupted inhibitory and excitatory synaptic functions in *PV-Cre; GAD67^{flox/+}* mice. (a and b) The input–output relationships of GABA_A IPSCs of controls (Con, *n* = 11) and *PV-Cre; GAD67^{flox/+}* mice (Het, *n* = 11). GABA_A receptor-mediated synaptic responses were smaller in *PV-Cre; GAD67^{flox/+}* mice than in controls. *P* = 0.0003 by two-way repeated measures ANOVA. **P* < 0.05 by Bonferroni *post-hoc* test. Representative traces are shown in b. (c and d) The input–output relationships of AMPA EPSCs of controls (Con, *n* = 17) and *PV-Cre; GAD67^{flox/+}* mice (Het, *n* = 15). *P* = 0.74 by two-way repeated measures ANOVA. Representative traces are shown in d. (e) AMPA receptor-mediated EPSCs (downward traces) and NMDA receptor-mediated EPSC (upward traces) were recorded at membrane potentials of –90 and +40 mV, respectively. (f) The ratio of amplitudes of NMDA EPSCs to those of AMPA EPSCs was similar between the two genotypes (*P* = 0.26). (g) NMDA EPSCs were recorded at membrane potentials between +40 and –90 mV. (h) The current amplitudes were normalized to the value obtained at +40 mV in each cell, and the values were then averaged for all cells. NMDA EPSCs in *PV-Cre; GAD67^{flox/+}* mice displayed significantly smaller synaptic currents in the *I*–*V* curve at membrane potentials between –40 and –20 mV in *PV-Cre; GAD67^{flox/+}* mice (Con, *n* = 9; Het, *n* = 8; ***P* < 0.005 by two-way repeated measures ANOVA). ****P* < 0.001 by Bonferroni *post-hoc* test. (i) Representative maximum intensity projection images of secondary apical dendrites of pyramidal neurons from a control and *PV-Cre; GAD67^{flox/+}* mice. Scale bar, 3 μm. (j) Quantification of secondary dendrite spine density in control (11 ± 1 spines per 10 μm, *n* = 13) and *PV-Cre; GAD67^{flox/+}* (16 ± 1 spines per 10 μm, *n* = 12) neurons (*P* < 0.05). ANOVA, analysis of variance; GAD67, glutamate decarboxylase 67; NS, not significant; PV, parvalbumin.

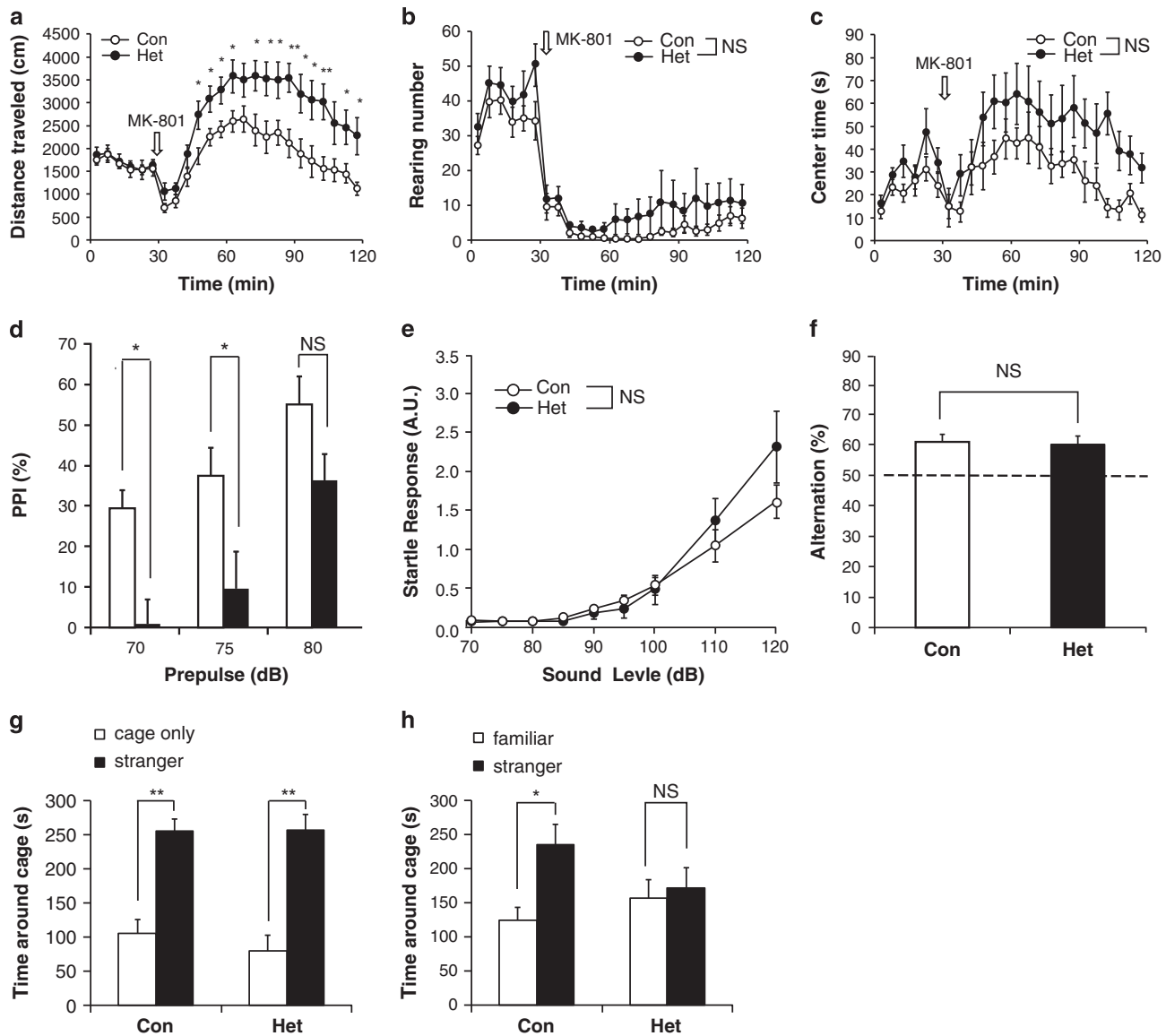


Figure 4 Schizophrenia-related behaviors in *PV-Cre; GAD67^{flox/±}* mice. (a–c) Normal spontaneous locomotion (1–30 min; $P=0.21$) but locomotor stimulatory effect of MK-801 in an open field was significantly greater in *PV-Cre; GAD67^{flox/±}* mice compared with controls (Con, $n=10$; Het, $n=9$; 31–120 min, $P<0.05$, two-way repeated measures ANOVA, * $P<0.05$, ** $P<0.01$ by Shaffer's *post-hoc* test). Distance traveled (a), rearing number (b), and time spent in the center area (c). (d) Prepulse inhibition for the 70 and 75 dB prepulse sound levels followed by 120 dB startle stimulus was significantly impaired in *PV-Cre; GAD67^{flox/±}* mice compared with controls ($P<0.005$, two-way repeated measures ANOVA; $P<0.05$ for 70 dB; $P<0.05$ for 75 dB, and $P=0.31$ for 80 dB by Holm's *post-hoc* test). (e) Acoustic startle responses were not different between genotypes ($P=0.47$, Two-way repeated measures ANOVA). (f) Y-maze spontaneous alternation task. There was no significant difference between the two genotypes (Con, $n=18$; Het, $n=15$; $P=0.55$). (g) In the sociability test, *PV-Cre; GAD67^{flox/±}* mice spent time interacting with the novel targets comparable to controls (paired *t*-test, Con; $P<0.0005$, Het; $P<0.001$, respectively). (h) In the social novelty test, *PV-Cre; GAD67^{flox/±}* mice spent significantly less time interacting with the novel targets than controls (paired *t*-test, Con, $P<0.005$; Het, $P=0.75$). Con, $n=18$; Het, $n=15$. ANOVA, analysis of variance; GAD67, glutamate decarboxylase 67; NS, not significant; PV, parvalbumin.

behavioral alterations related to schizophrenia. Furthermore, we showed that the number of PV neurons in the cerebral cortex was decreased, inhibitory transmission was reduced, and the properties of NMDA receptor-mediated synaptic responses in the hippocampus were altered in *PV-Cre; GAD67^{flox/±}* mice. These results suggest that haploinsufficiency of the GAD67 gene in a subset of GABAergic neurons, including PV neurons, gives rise to schizophrenia-related endophenotypes, thus supporting the hypothesis that GAD67 deficiency in PV neurons is involved in disease pathogenesis.

We note that most schizophrenia cases appear to be a polygenic rather than a single gene disorder with several risk genes of small effect (Insel, 2010). However, several genetically modified mouse models for genes relevant to schizophrenia and pharmacological rodent models for schizophrenia have suggested that they commonly impair GAD67 expression, especially in PV-positive subtypes: cortical interneuron-specific GluN1-knockout mice exhibit GAD67 reduction in cortical GABAergic neurons including PV-positive subtypes (Belforte *et al*, 2010); ketamine

administration, based on the hypoglutamatergic hypothesis, decreases GAD67 and PV expression in the cerebral cortex (Behrens *et al*, 2007); methylazoxymethanol acetate administration on gestational day 15, based on the developmental disruption hypothesis, reduces the expression of PV-containing interneurons in the cerebral cortex (Lodge *et al*, 2009); repeated amphetamine administration following long-term withdrawal periods, based on the endogenous dopamine sensitization hypothesis, induces a downregulation of GAD67 immunoreactivities in the hippocampus, prefrontal cortex, thalamus, and amygdala (Peleg-Raibstein *et al*, 2008). Our results suggest that in these model rodents, GAD67 deficiency contributes to the development of schizophrenia-like phenotypes and is a common pathway in schizophrenia pathogenesis. In addition, there are a number of rodent models of schizophrenia showing PV neuron deficiency: functional DISC1 deficits decrease PV-positive neurons (Hikida *et al*, 2007); PV neuron-specific ErbB4-knockout mice exhibit schizophrenia-relevant phenotypes (Wen *et al*, 2010); prenatal infection with the viral mimic polyriboinosinic–polyribocytidylic acid, a model based on epidemiological studies, reduces the density of PV-positive neurons (Ducharme *et al*, 2012). Based on these reports and together with our data, we speculate that GAD67 deficiency in PV-positive interneurons in these rodent models relates to the development of their schizophrenia-relevant phenotypes, *albeit* with heterogeneous mechanisms for the dysfunction of PV-positive neurons.

How could GAD67 deficiency in a subset of GABAergic neurons induce certain schizophrenia-like phenotypes in mice? *PV-Cre; GAD67^{fllox/+}* mice show reduced GAD67 expression and decreased GABA_A receptor-mediated inhibitory synaptic transmission without affecting AMPA receptor-mediated excitatory synaptic transmission. *PV-Cre; GAD67^{fllox/+}* mice also have an altered *I–V* relationship of NMDA EPSCs and exhibit increased number of dendritic spine density in the pyramidal neurons of the hippocampal CA1 region. The properties of the NMDA receptors in *PV-Cre; GAD67^{fllox/+}* mice are similar to those in the neonatal hippocampal CA1 region (Kirson *et al*, 1999). Because GABA regulates excitatory synapse formation (Ben-Ari *et al*, 2004), these results imply that reduced GABA expression levels in *PV-Cre; GAD67^{fllox/+}* mice disturb developmental pruning (Huttenlocher and Dabholkar, 1997) and the maturation of excitatory synaptic functions, even though dendritic spine density on prefrontal cortical pyramidal neurons in subjects with schizophrenia are decreased (Glantz and Lewis, 2000). One of the characteristic properties of NMDA receptors is the blockade of the receptor channel by extracellular Mg²⁺ block at negative membrane potentials and it has a critical role in activity-dependent modifications of excitatory synapses that are observed during long-term potentiation and synapse formation (Hunt and Castillo, 2012). The timing of Mg²⁺ unblock is critical in determining the contribution of NMDA receptors to spike generation (Schiller and Schiller, 2001). The low voltage dependence of NMDA receptors in mutant mice would affect the time course of Mg²⁺ unblock to detect coincident pre and postsynaptic activity and may impede excitatory synaptic maturation and plasticity in the adult hippocampus. Taken together, the balance between excitation and inhibition in CA1 pyramidal neurons is shifted toward excitation in the

mutant mice. In fact, enhanced hippocampal activity has been reported in animal models and schizophrenia patients (Lodge *et al*, 2009; Schobel *et al*, 2009) and correlates with enhanced dopamine neuron activity and positive symptoms (Blahe *et al*, 1997; Legault and Wise, 1999). The *PV-Cre; GAD67^{fllox/+}* mice exhibited enhanced MK-801-induced hyperlocomotion in the open field test. Because hyperlocomotor activity is commonly considered a behavior of increased dopaminergic tone (Laruelle and Abi-Dargham, 1999), the motor stimulatory effects of MK-801 in *PV-Cre; GAD67^{fllox/+}* mice might be associated with subcortical dopamine hyperfunction. To clarify this issue, further examination would be required.

Homozygous *PV-Cre; GAD67^{fllox/fllox}* mice showed increased GAD65 and PV expression and increased number of PNN-positive cells, and some of them exhibited epileptic seizures. These results are inconsistent with the following results of postmortem brain studies of patients with schizophrenia (Beasley and Reynolds, 1997; Guidotti *et al*, 2000; Hashimoto *et al*, 2008; Mauney *et al*, 2013; Todtenkopf and Benes, 1998; Woo *et al*, 1997; Zhang and Reynolds, 2002): (1) GAD65 mRNA and protein expression levels are unaffected or downregulated in the cerebral cortex of schizophrenic patients; (2) mRNA levels of PV in the cerebral cortex are lower in schizophrenic patients than control subjects; (3) lower PV mRNA levels have been found in a number of animal models for schizophrenia; and (4) the densities of PNNs are decreased in the prefrontal cortex in schizophrenia compared with normal control subjects. Because the regulation of PV-synaptic innervations during the maturation of inhibitory circuits is dependent on GAD67-mediated GABA synthesis (Chattopadhyaya *et al*, 2007), the dysfunction of PV inhibitory circuits cause a further excitation of pyramidal neurons. This effect could contribute to epileptic seizure generation and induce compensatory responses in homozygous *PV-Cre; GAD67^{fllox/fllox}* mice. In fact, such seizure activity has been known to involve compensatory changes in gene expression including the upregulation of GAD65 and PV expression in the hippocampus (Esclapez and Houser, 1999; Hwang *et al*, 2007). Therefore, since GAD67 mRNA and protein levels are moderately reduced, not completely lost, in schizophrenia relative to matched control subjects (Guidotti *et al*, 2000; Hashimoto *et al*, 2003; Torrey *et al*, 2005; Volk *et al*, 2000), heterozygous *PV-Cre; GAD67^{fllox/+}* mice are a new, potential rodent model of schizophrenia that will be useful for further understanding schizophrenia pathogenesis.

In conclusion, we describe a new association between GAD67 deficiency in a mouse model and the results from postmortem brain studies of schizophrenia patients. Haploinsufficiency of the GAD67 gene in a subset of GABAergic neurons, primarily in PV neurons, gives rise to schizophrenia-related endophenotypes and alterations in both excitatory and inhibitory synaptic functions. These findings are consistent, in several respects, with results of other reports (Brown *et al*, 2015; Lazarus *et al*, 2013). Therefore, our findings support the hypothesis that GAD67 deficiency is a possible common pathway that induces the pathogenesis of schizophrenia and provide a novel link between GAD67 deficiency and impaired NMDA-mediated synaptic transmission.

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The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

KF, HM, and YY designed the research? KF and HM performed the research; TK, MM, NT, and YY contributed reagents/analytic tools; CT and NT generated the *PV-Cre* mice; RK generated the *VGAT-floxed STOP-tdTomato* mice; KF and HM analyzed the data; and KF, HM, and YY prepared the manuscript, which was approved by all authors.

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