ORIGINAL ARTICLE

Selective loss of parvalbumin-positive GABAergic interneurons in the cerebral cortex of maternally stressed *Gad1*-heterozygous mouse offspring

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Exposure to maternal stress (MS) and mutations in *GAD1*, which encodes the γ-aminobutyric acid (GABA) synthesizing enzyme glutamate decarboxylase (GAD) 67, are both risk factors for psychiatric disorders. However, the relationship between these risk factors remains unclear. Interestingly, the critical period of MS for psychiatric disorders in offspring corresponds to the period of GABAergic neuron neurogenesis and migration in the fetal brain, that is, in the late stage of gestation. Indeed, decrement of parvalbumin (PV)-positive GABAergic interneurons in the medial prefrontal cortex (mPFC) and hippocampus (HIP) has often been observed in schizophrenia patients. In the present study, we used GAD67-green fluorescent protein (GFP) knock-in mice (that is, mice in which the *Gad1* gene is heterozygously deleted; GAD67[+/GFP]) that underwent prenatal stress from embryonic day 15.0 to 17.5 and monitored PV-positive GABAergic neurons to address the interaction between *Gad1* disruption and stress. Administration of 5-bromo-2-deoxyuridine revealed that neurogenesis of GFP-positive GABAergic neurons, but not cortical plate cells, was significantly diminished in fetal brains during MS. Differential expression of glucocorticoid receptors by different progenitor cell types may underlie this differential outcome. Postnatally, the density of PV-positive, but not PV-negative, GABAergic neurons was significantly decreased in the mPFC, HIP and somatosensory cortex but not in the motor cortex of GAD67[+/GFP] mice. By contrast, these findings were not observed in wild-type (GAD67^{+/+}) offspring. These results suggest that prenatal stress, in addition to heterozygous deletion of *Gad1,* could specifically disturb the proliferation of neurons destined to be PV-positive GABAergic interneurons.

Translational Psychiatry (2014) **4,** e371; doi:10.1038/tp.2014.13; published online 11 March 2014

Keywords: GAD67; glucocorticoid receptor; neurogenesis; parvalbumin; prenatal stress; schizophrenia

INTRODUCTION

Environmental factors, such as maternal stress (MS), have been linked to psychiatric disorders in offspring, including schizophrenia.[1,2] In addition, psychological stress in pregnant women is associated with childhood adjustment disorders in their offspring.[3] In support of temporal specificity for the effects of stress on long-term brain developmental disorders, an epidemiological study reported a significant association between MS experienced during pregnancy and an increased risk of schizophrenia in offspring.[4] Comparable behavioral disruptions are observed in animals that have experienced prenatal stress.[5,6]

During sensitive periods of development, adverse events such as stress can readily trigger epigenetic alterations, which can adversely affect physiological function and behavior in adulthood.[7,8] The fetal period involves dynamic development of the cerebral cortex and the hippocampus (HIP), and excess stress hormone levels can cause cell loss in these sensitive brain areas.[9] Interestingly, the period of highest vulnerability to prenatal stress has been matched to the peak period of proliferation and migration of neurons during hippocampal and cortical development.[10]

Whereas differentiated excitatory glutamatergic pyramidal neurons originate from the ventricular zone (VZ) of the dorsal telencephalon form the cortical plate (CP),[11] GABAergic neurons are born in the VZ of the ventral telencephalon in the medial ganglionic eminence (MGE).[12–14] Postmitotic MGE-derived neurons exit proliferative domains through intricate but highly stereotyped pathways, migrating dorsally toward the neocortex and caudally to the HIP.[15]

Strong evidence indicates that the GABA-related system is impaired in schizophrenia.[16–19] In particular, studies have indicated reductions in cortical GABA content[20] and the activity of glutamate decarboxylase (GAD) 67,[21–23] a GABA synthesizing enzyme. In childhood-onset schizophrenia cases, Addington *et al.*[24] observed significant overtransmission of alleles at several adjacent SNPs in the 5′ region of the *GAD1* gene encoding GAD67, suggesting that *GAD1* may be a fairly common genetic risk factor for schizophrenia. GABAergic interneurons are classified by diverse molecular, morphological and physiological properties.[25] Notably, GABA-related abnormalities in schizophrenia and related disorders are largely exclusive to the basket and chandelier types of neurons expressing parvalbumin (PV).[26–28]

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Although the pathogenesis and pathophysiology of schizophrenia and other psychiatric disorders involve multiple factors, such as MS (or prenatal stress), impairment of GAD67 and loss of PV-positive GABA neurons, the relationship among these factors is unknown. We previously established an MS model using GAD67-green fluorescent protein (GFP) knock-in mice.[29] In GAD67-GFP knock-in mice, GFP was specifically expressed in GABAergic neurons under the control of the endogenous *Gad1* promoter, and these knock-in mice have helped to elucidate the development of GABAergic neurons.[30,31] In addition, the protein expression level of GAD67 was shown to be reduced in heterozygous GAD67-GFP knock-in (GAD67$^{+/GFP}$) mice because of disruption of the endogenous *Gad1* gene.[32] Therefore, in addition to its consistent labeling of GABAergic neurons, the GAD67$^{+/GFP}$ mouse is useful for the study of decreased GAD67 levels with *Gad1* gene disruption. Because this model shows higher vulnerability to MS in GAD67$^{+/GFP}$ than in wild-type (GAD67$^{+/+}$) fetuses,[29] we hypothesized that MS with *GAD1* abnormalities may affect neurogenesis and/or migration of PV-positive GABAergic neurons. Using this model, we investigated the gene–environment interaction between MS and fetal *Gad1* deletion as an interactive risk factor for psychiatric disorders, including schizophrenia. Our results confirm that GAD67$^{+/GFP}$ mice exposed to MS show an anatomical phenotype of PV-positive GABAergic neurons similar to that observed in psychiatric and autistic patients.

MATERIALS AND METHODS

Animals
All procedures were conducted in accordance with the guiding principles of the NIH, under the review and approval of the Guide for the Care and Use of Laboratory Animals of Hamamatsu University School of Medicine, Japan. Every effort was made to minimize the number and suffering of the animals used. The generation of GAD67-GFP (Δneo) mice has been described previously.[30] In brief, as a consequence of replacement of the endogenous *Gad1* gene by the *gfp* gene, GAD67 protein and ambient GABA levels in the GAD67$^{+/GFP}$ brain are reduced by ~ 50% relative to the wild-type (GAD67$^{+/+}$) brain.[32,33] In the present study, female GAD67$^{+/+}$ mice (Japan SLC, Hamamatsu, Japan) were placed overnight with male (>9 weeks) GAD67$^{+/GFP}$ mice in a cage under a 12-h light–dark cycle (lights off from 1800 to 0700 hours). The day when a plug was identified was defined as embryonic day (E) 0. For postnatal experiments, mothers were changed to naive surrogate mothers with the same delivery date at postnatal day (P) 0. Pups were bred until P21 with their surrogate mother.

Maternal restraint-and-light stress
The procedure of maternal restraint-and-light stress was described previously.[29] In brief, the stress procedure was performed three times a day for 45 min per session (0830–0915 hours, 0030–1315 hours and 1630–1715 hours) from E15.0 to E17.5 with a transparent plastic tube with a diameter of 3 cm.

BrdU administration
Pregnant mice were intraperitoneally injected with 5-bromo-2-deoxyuridine (BrdU, 50 mg kg^{-1}; Sigma-Aldrich, Tokyo, Japan) dissolved in 0.1 M phosphate-buffered saline (PBS) once at E15.0 or E12.0.

Immunohistochemistry
Pregnant mothers were deeply anesthetized with pentobarbital sodium (50 mg kg^{-1}, intraperitoneally), and fetuses were dissected out after the final stress session. GAD67$^{+/GFP}$ fetuses were perfused intracardially with fixative solution consisting of 4% (w/v) paraformaldehyde in 0.1 M PBS. Coronal sections (25 μm-thick) were treated with 2 N HCl for 1 h at 37 °C, blocked with 10% (v/v) horse serum in PBS and 0.5% (v/v) Triton X-100 (PBST) and incubated with primary antibodies against GFP (A11122, Life Technologies, Tokyo, Japan, rabbit polyclonal 1:1000) and BrdU (B44, Becton Dickinson, Franklin Lakes, NJ, USA, mouse monoclonal, 1:400) overnight at 4 °C. Sections were then incubated with secondary antibodies

(AlexaFluor 488 anti-rabbit, AlexaFluor 546 anti-mouse, Life Technologies, Eugene, OR, USA) for 2 h at room temperature.

For determination of glucocorticoid receptor (GR) distribution and cell proliferation, sections from GAD67$^{+/+}$ fetuses were treated with 100% (v/v) methanol for 10 min and then incubated with an anti-GR antibody (ab9568, Abcam, Tokyo, Japan, mouse monoclonal 1:200) and anti-Ki-67 antibody (CRM325, Biocare Medical, Concord, CA, USA, rabbit monoclonal 1:100) overnight at 4 °C. After washing, sections were incubated with secondary antibodies for 2 h at room temperature.

P21 male GAD67$^{+/GFP}$ and GAD67$^{+/+}$ mice were anesthetized and intracardially perfused with fixative solution. Brain sections were prepared as above, and GFP and BrdU double immunostaining was performed. For double immunostaining of GFP and PV, sections were first immunostained for GFP. For double immunostaining of GAD67 and PV in P21 GAD67$^{+/+}$ mice, anti-GAD67 (MAB5406, Millipore-Japan, Tokyo, Japan, mouse monoclonal, 1:2000) and AlexaFluor 488 anti-mouse antibodies were applied first, followed by incubation with the anti-PV antibody (PC255L, Calbiochem, San Diego, CA, USA, rabbit polyclonal, 1:200) overnight at 4 °C and the secondary antibody for 2 h at room temperature.

Cell counting
We attempted to ensure unbiased counting[34,35] as follows. In brief, to prevent overcounting, we configured a single rectangular region of interest within each brain region to prevent double counting of cells. The section thickness was consistent, and multiple sections were counted from the same brain sample. Tissue sections were scanned using an inverted microscope system (BZ-9000, KEYENCE, Osaka, Japan), and the fluorescence of the secondary antibodies was separately detected. Cells were analyzed using an image analyzer (BZ-II Analyzer, KEYENCE) with measurement (BZ-H1M, KEYENCE) and cell-counting (BZ-H1C, KEYENCE) software. Designated cell types were recognized by a software algorithm that disregards noise outside of the fluorescence intensity window or signals that are too large or too small to meet the criteria for cell recognition. The conditions of the cell count can be saved across assays, hence leading to high reproducibility between sections. The BrdU-positive cells (red), PV-positive cells (red), GFP-positive cells (green), GAD67-positive cells (green) and co-labeled cells (yellow) were counted in the region of interests. Cell densities were calculated in each region of interest. For analysis of cell distribution in the cerebral cortex, the % distribution of cells in each brain region was calculated. One to three sections were arbitrarily selected from each animal brain.

Injection of dexamethasone and mifepristone into fetal lateral ventricles
Dexamethasone (DEX; 1 mM) and mifepristone (MIFE; 1 mM, Sigma-Aldrich) were dissolved in *N*-methyl-2-pyrrolidone (NMP; Wako Pure Chemical Industries, Wako, Japan), and adsorbed on an absorbent polymer nanoparticle poly(lactic-co-glycolic) acid (PLGA, Wako) (1 mg PLGA/4 μl NMP).[36–38] The uterine horns were exposed under anesthesia, and the drug-absorbed PLGA (0.5 μl) was bilaterally injected into the lateral ventricles of GAD67$^{+/+}$ mice at E15.0 using borosilicate glass capillaries (GC100F-10, Harvard Apparatus, Holliston, MA, USA). Assuming a 0.8 g water weight for the fetus, the concentration was estimated as 1.25 nmol ml^{-1}, and 20–30% of the PLGA-adsorbed DEX was expected to be released in 2 days.[36] The maternal abdomen was then sutured and allowed to recover. Brain sections were prepared as described above for E17.5 fetuses.

TUNEL (TdT-mediated dUTP nick-end labeling) assay
The TMR red *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany) was used in accordance with the manufacturer's protocol. For a positive control, sections were incubated for 15 min at 37 °C in proteinase K, followed by treatment with DNase I (10 U ml^{-1}) for 30 min at 37 °C.

Statistical analysis
Fetal body weight, maternal serum corticosterone (CORT) levels and the density of immunopositive cells in each area were analyzed using Student's *t*-test. The Pearson χ^2-test was used to evaluate the distribution pattern of migrating cells: tangentially migrating BrdU (injected at E12.0 or 15.0) and GFP double-positive GABAergic cells and radially migrating BrdU-positive and GFP-negative CP cells born during MS. $P < 0.05$ was considered to be significant.

RESULTS

Effect of MS on the proliferation and radial migration of CP cells in the GAD67$^{+/GFP}$ fetal brain

Pregnant GAD67$^{+/+}$ mice giving rise to GAD67$^{+/GFP}$ fetuses were given restraint-and-light stress, and BrdU was used to label cells dividing during this period. BrdU was injected immediately before starting the MS (Figure 1a). After the final session of MS, the brains of GAD67$^{+/GFP}$ fetuses were examined for CP cells and GABAergic neurons generated during MS (Figures 1b and c).

The density and distribution pattern of CP cells, that is, BrdU (+)/GFP (−) cells, in the CP, subplate (SP), intermediate zone (IZ), IZ/subventricular zone (SVZ) and VZ of the cerebral cortex were investigated. Neither the regional density nor the distribution pattern of CP cells was altered by MS (Figures 1d and e). These

results indicate that proliferation and radial migration of CP cells were unaffected by prenatal stress.

Effect of MS on the population of GABAergic neurons in the GAD67$^{+/GFP}$ fetal cerebral cortex

GABAergic neurons were classified according to GFP expression, and BrdU (+)/GFP (+) cells were counted in the cerebral cortex (Figures 1b and c) of control and stressed GAD67$^{+/GFP}$ fetuses. BrdU (+) GABAergic neurons were decreased in the IZ, IZ/SVZ and VZ of the stressed fetal cerebral cortex (IZ, control 289 ± 33 cells mm^{-2}, stress 78 ± 30 cells mm^{-2}, $F_{1,6} = 1.22$, $P < 0.001$; IZ/SVZ, control 1677 ± 182 cells mm^{-2}, stress 564 ± 110 cells mm^{-2}, $F_{1,6} = 2.74$, $P < 0.001$; VZ, control 469 ± 67 cells mm^{-2}, stress 119 ± 22 cells mm^{-2}, $F_{1,6} = 9.28$, $P < 0.01$). The density of the BrdU
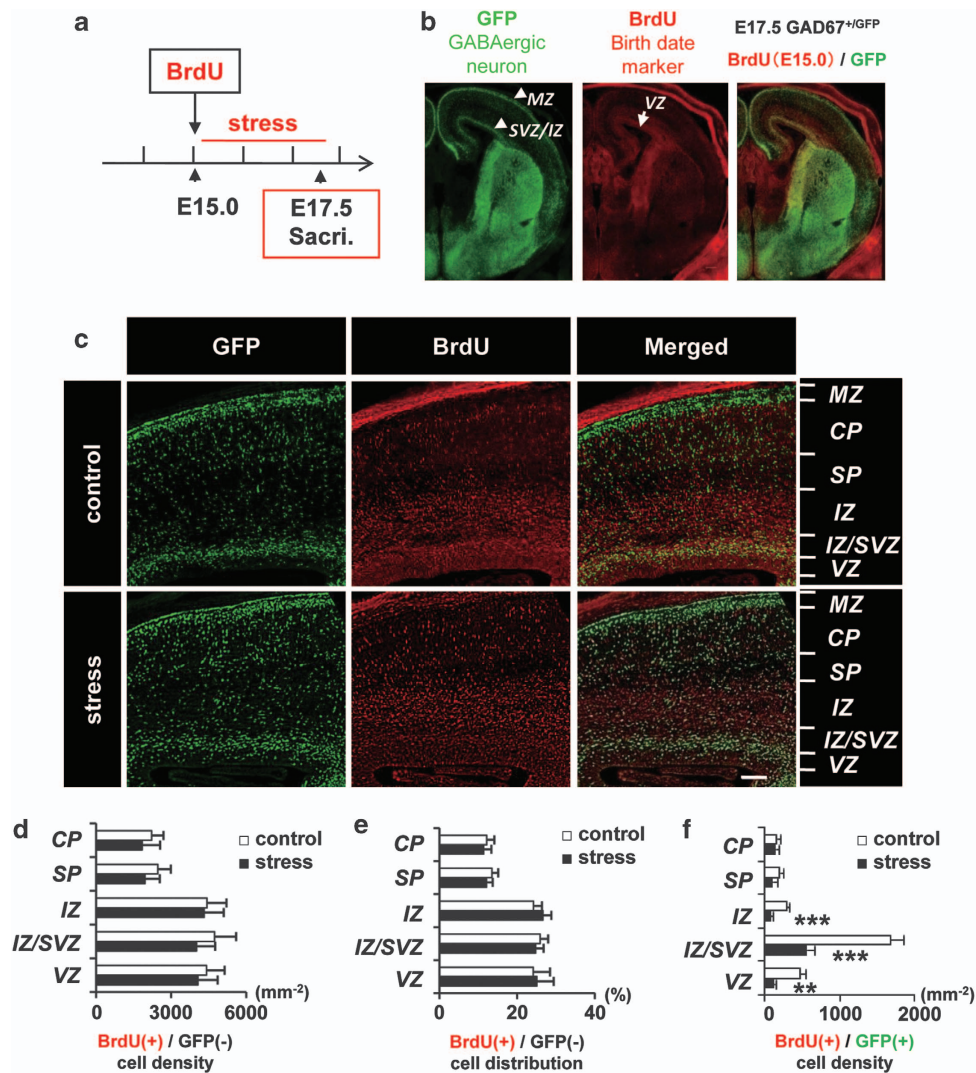
Figure 1. The density of γ-aminobutyric acidergic (GABAergic) neurons, but not that of cortical plate cells, born during the maternal stress (MS) decreased in the fetal cerebral cortex. (**a**) Time course of the MS and BrdU administration. BrdU was administered to pregnant mice at E15.0, before the first stress session. GAD67$^{+/GFP}$ fetuses were dissected out at E17.5 after the final session of stress. (**b**) Orientation of the fetal forebrain. Fluorescence images of green fluorescent protein (GFP; green, GABAergic neuron) and BrdU (red, cells born at E15.0) in control and stressed mice. Double-positive cells (yellow) are GABAergic neurons born during the MS. (**c**) Higher-magnification image of control and the stressed fetal neocortex immunostained with GFP and BrdU antibodies, and their merged image. CP, cortical plate; IZ, intermediate zone; IZ/SVZ, intermediate zone/subventricular zone; MZ, marginal zone; SP, subplate; VZ, ventricular zone. IZ is divided into IZ/SVZ and the rest of IZ because IZ/SVZ is the main route of tangential migration.[31] Bar = 100 μm. (**d**) The density of BrdU-positive CP cells, that is, BrdU(+) and GFP(−), in each neocortical area of maternally stressed fetuses was not affected ($P > 0.05$, t-test in each area). (**e**) The distribution of BrdU-positive CP cells in each neocortical area was not significantly different between control and stressed mice. ($P = 0.99$, Pearson's χ^2-test). (**f**) The density of BrdU-positive GABAergic neurons, that is, BrdU(+) and GFP(+), was significantly decreased in IZ, IZ/SVZ and VZ of the stressed fetal cerebral cortex. **$P < 0.01$; ***$P < 0.001$, t-test in each area. Bars represent the mean ± s.e.m., $n = 11$ sections from four pups in each group (**d**–**f**).
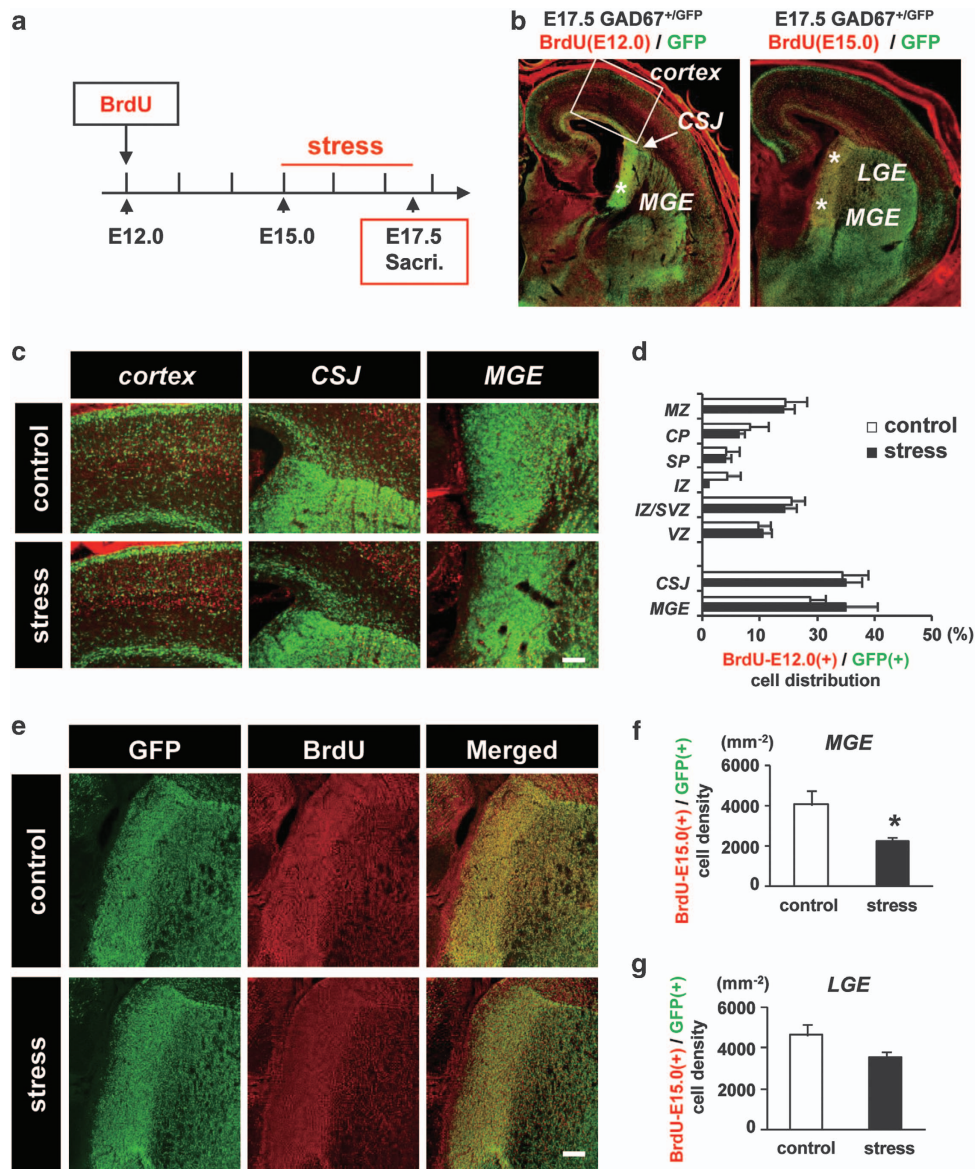
Figure 2. The number of newly born γ-aminobutyric acidergic (GABAergic) neurons decreased in the medial ganglionic eminence (MGE) following maternal stress (MS). (**a**) Time course of MS and BrdU injection. BrdU was administered to pregnant mice at E12.0. MS was performed from E15.0 to 17.5, and fetuses were dissected out at E17.5. (**b**) An E17.5 GAD$^{+/GFP}$ fetal forebrain injected with BrdU at E12.0 (left) or E15.0 (right). CSJ, corticostriatal junction; LGE, lateral ganglionic eminence. (**c**) Higher-magnification image of the neocortex showing CSJ and MGE of control and stressed fetal brains in **b** (left). (**d**) Distribution of BrdU-positive (injected at E12.0) GABAergic neurons. The % distribution of BrdU-E12.0(+)/GFP(+) cells in each area of the neocortex and ganglionic eminence (GE) was not significantly different between control and stressed fetuses ($P = 0.85$, Pearson's χ^2-test; control: $n = 15$ sections from five pups, stress: $n = 19$ sections from five pups). Bars represent the mean ± s.e.m. (**e**) Higher-magnification image of the section shown in **b** (right). MGE and LGE immunostained for green fluorescent protein (GFP) and BrdU (injected at E15.0) and their merged image. Bar = 100 μm. (**f**) The density of BrdU-positive GABAergic neurons, BrdU-E15.0 (+)/GFP(+), was significantly decreased by MS in the MGE. (*$P < 0.05$, t-test; control, $n = 11$ sections from seven pups; stress, $n = 18$ sections from seven pups). (**g**) In the LGE, the density of BrdU-positive GABAergic neurons appeared to decrease following MS, but the decrease was not significant ($P = 0.10$, t-test; control, $n = 11$ sections from seven pups; stress, $n = 18$ sections from seven pups). Error bars represent the s.e.m.

and GFP double-positive cells in the control and stressed fetal brains showed no significant differences in the CP and SP (Figure 1f). The total density of BrdU (−)/GFP (+) cells in the fetal cerebral cortex was not altered by MS (data not shown). These data indicate that MS decreases the number of neocortical GABAergic neurons born during the stress period.

Effect of MS on the tangential migration of GABAergic neurons in GAD67$^{+/GFP}$ fetuses

To determine whether prenatal stress retarded tangential migration and resulted in a decrease in GABAergic neurons in the

neocortex, we examined the distribution of GABAergic neurons born before the stress period. BrdU was injected at E12.0, and MS was performed from E15.0 to E17.5 (Figure 2a). The distribution pattern of BrdU-GFP double-positive cells in each area of the fetal ganglionic eminence (GE) and the neocortex was unaffected by MS (Figure 2b), indicating that tangential migration was not affected by prenatal stress.
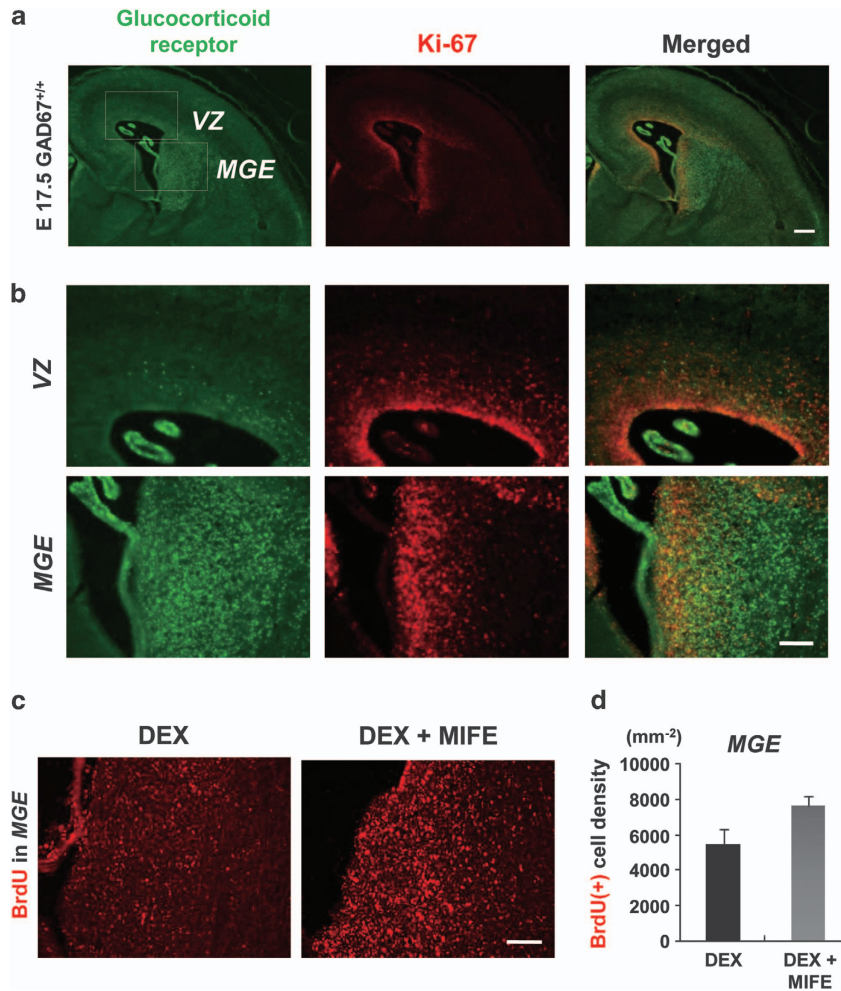
Figure 3. Progenitor γ-aminobutyric acidergic (GABAergic) interneurons in the medial ganglionic eminence (MGE), but not cortical plate (CP) cells in the ventricular zone (VZ), express glucocorticoid receptor (GR). (**a**) Immunostaining of GR (green) and Ki-67 (red) and their merged images from E17.5 fetal brain; $n = 3$ sections from three pups. (**b**) Higher-magnification image of (**a**). Many proliferating cells in the MGE, but not in the VZ, expressed GR. Bars $= 50$ μm. (**c**) BrdU immunostaining at E17.5 in the MGE from either Dexamethasone (DEX) (−) (left) or DEX and mifepristone (MIFE)-treated (right) E15.0 fetuses. (**d**) The density of BrdU-positive cells in the MGE in DEX and MIFE-treated fetuses was higher than that in DEX-treated fetuses. $P = 0.077$, *t*-test. DEX, $n = 3$ sections from three pups; DEX+MIFE, $n = 4$ sections from three pups.

Effect of MS on the generation of GABAergic neurons in the ganglionic eminence of GAD67+/GFP fetuses

We investigated the number of BrdU (injected at E15.0) and GFP double-positive cells in the fetal GE (Figure 2e). The number of BrdU-positive GABAergic neurons decreased significantly in the MGE, but not in the lateral ganglionic eminence (LGE; Figures 2f and g; MGE, control 4156 ± 546 cells mm^{-2}, stress 2373 ± 270 cells mm^{-2}, $F_{1,12} = 10.6$, $P < 0.05$; LGE, control 4591 ± 412 cells mm^{-2}, stress 3795 ± 370 cells mm^{-2}, $F_{1,12} = 5.80$, $P = 0.10$). These data indicate that the number of GABAergic neurons generated in the fetal MGE was significantly decreased during MS.

## Distribution pattern of proliferating cells and GRs in fetal brains

Because maternally stressed fetuses respond by increasing CORT (Supplementary Figure 1d)[29], we investigated the localization of GRs in proliferating cells (Figures 3a and b) to determine why only GABAergic precursors were vulnerable to MS ($n = 3$). Fetal brains were immunostained for GR and Ki-67. Although few GR and Ki-67 double-positive cells were present in the VZ, many double-positive cells were observed in the MGE. These data indicate that the proliferating cells in the MGE, but not in the VZ, can be affected by CORT via GR.

Effect of GR activation on cell proliferation in the MGE

To investigate whether increased CORT (Supplementary Figure 1) is responsible for impairment of neuronal proliferation in the MGE, we administered the GR agonist DEX (1 mM) or DEX together with the GR antagonist MIFE (1 mM) absorbed on PLGA into the lateral ventricles of GAD67+/+ fetuses at E15 (Figure 3c). Assuming that the bioactivity of DEX at GRs is 20–30 times higher than that of endogenous corticosterone, the total steroid level after DEX injection should be comparable to that in the stressed fetuses. Two days later, the cell density increased by 40% when the antagonist was co-applied with a GR agonist (Figure 3d; DEX, 5479 ± 821 cells mm^{-2}, DEX+MIFE, 7618 ± 570 cells mm^{-2}, $F_{1,5} = 11.56$, $P = 0.077$). Although statistical significance was not established, CORT may disturb cell proliferation via GR in the MGE (see Discussion).

Evaluation of cell death in the MGE in GAD67+/GFP fetuses exposed to MS

We used the TUNEL assay to determine whether the decreased number of BrdU (+)/GFP (+) cells was caused by cell death, (Supplementary Figure 2). Several TUNEL-positive cells were observed in the neocortex of both control ($n = 3$) and stressed ($n = 4$) fetal brains (Supplementary Figure 2b), whereas few cells
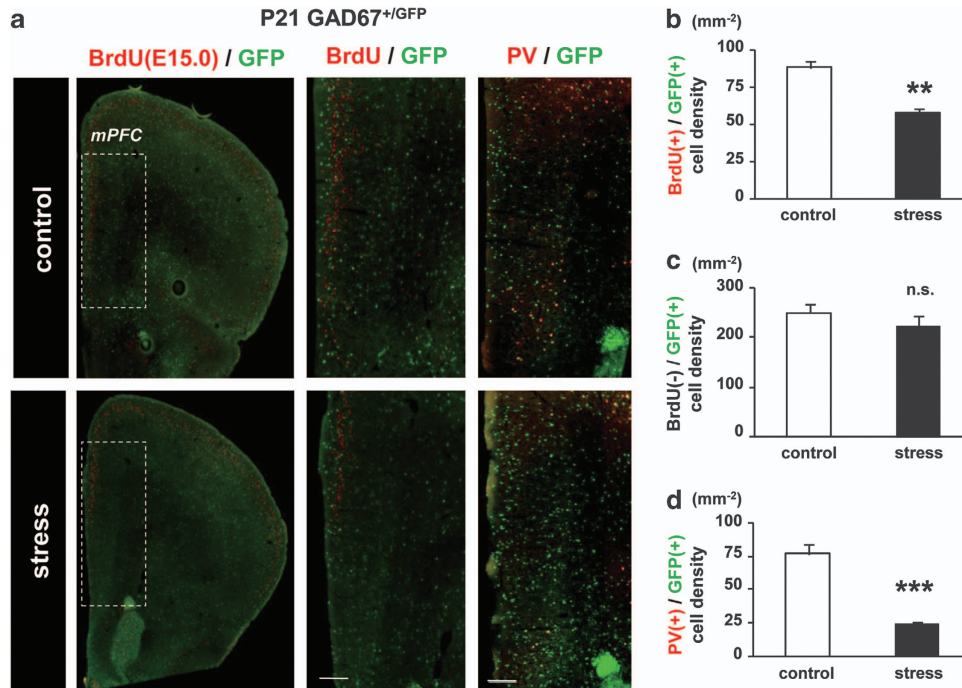
Figure 4. Loss of parvalbumin (PV)-positive γ-aminobutyric acidergic (GABAergic) interneurons in the medial prefrontal cortex (mPFC) of maternally stressed P21 GAD67[+/GFP] offspring. (**a**) Region of analysis in the mPFC of a coronal section at P21 (left, dotted square). BrdU was injected at E15.0 immediately before maternal stress (MS). Middle panels are BrdU and green fluorescent protein (GFP) double-positive cells at higher magnification in the region indicated in the left panel (BrdU/GFP). Right panels (PV/GFP) show PV and GFP double-positive cells in the mPFC. Bars = 200 μm. Note the reduction in double-positive cells in stressed offspring. (**b**) Quantitative analysis of BrdU and GFP double-positive cells in the mPFC at P21. The density of BrdU(+)/GFP (+) cells from the stressed group was significantly lower than that of the control (**$P < 0.01$, *t*-test; control, $n = 6$ sections from three brains; stress, $n = 7$ sections from four brains). (**c**) Quantitative analysis of BrdU-negative and GFP-positive cells in the mPFC. There was no significant difference between control and stressed pups ($P = 0.68$, *t*-test; control, $n = 6$ sections from three brains; stress, $n = 7$ sections from four brains). (**d**) Quantitative analysis of PV and GFP double-positive cells in the mPFC at P21. The density of PV (+)/GFP (+) cells in the stress group was significantly lower than that of the control (***$P < 0.001$, *t*-test; control, $n = 5$ sections from three brains; stress, $n = 5$ sections from four brains). Error bars represent the s.e.m.

were observed in the MGE (Supplementary Figure 2c). However, TUNEL-positive cells were detected in positive control sections treated with DNase I ($n = 3$). Thus, the decrease in GABAergic neurons generated during MS was not caused by cell death.

**Anatomical outcome of MS in the brains of P21 GAD67[+/GFP] offspring**

We examined the outcome of the decrease in GABAergic neuronal proliferation in the fetal MGE in P21 male GAD67[+/GFP] offspring stressed during the fetal period. GABAergic interneurons generated during MS were studied in the medial prefrontal cortex (mPFC), an area involved in impaired cognition in schizophrenia[39] (Figure 4). The density of BrdU (injected at E15.0) and GFP double-positive cells was significantly decreased by exposure to MS (Figure 4b; control, 87.97 ± 4.71 cells mm^{-2}, stress, 61.95 ± 3.56 cells mm^{-2}, $F_{1,11} = 0.20$, $P < 0.01$). In contrast, the density of BrdU-negative and GFP-positive cells was not different between the control and stress groups (Figure 4c). These data indicate that impairment of GABAergic neurogenesis during exposure to MS resulted in a reduced number of GABAergic neurons in the mPFC of P21 offspring.

PV-containing interneurons are fundamental to the generation of normal synchronous activity and appear to be impaired in schizophrenia[40]. The number of PV-positive GABAergic interneurons was also significantly reduced in the mPFC (Figure 4d; control, 76.48 ± 7.16 cells mm^{-2}, stress, 24.00 ± 1.82 cells mm^{-2}, $F_{1,8} = 15.4$, $P < 0.001$) of GAD67[+/GFP] offspring. Similar reductions were observed in the HIP and the somatosensory cortex (S1), but not in the motor cortex (M1, Supplementary Figures 3a and b). There

were no significant differences in PV (−)/GFP (+) cell density in the control and stressed group in any region examined (Supplementary Figures 3a and c).

We next investigated the density of PV and GFP double-positive cells in subregions of the mPFC, HIP and S1 and found significant decreases in all layers of the mPFC (Figures 5a and c), in the CA1 region of the HIP (Figures 5b and e) and in layer III of the S1 (Figures 5b and g). In contrast, there were no significant differences in PV (−)/GFP (+) cell density in any of the evaluated regions (Figures 5d, f and h). These data indicate that the number of PV-positive GABAergic interneurons was specifically decreased in specific brain regions of *Gad1*-heterozygous P21 mice stressed during the fetal period.

Anatomical outcome of MS in the brain of P21 GAD67^{+/+} offspring

Finally, to address the question of whether heterozygous deletion of the *Gad1* gene contributes to the loss of PV-positive cells in MS-exposed mice, we examined GAD67^{+/+} offspring (Supplementary Figures 4a and b). No differences were found in the densities of PV and GAD67 double-positive cells between the control and stressed groups in the mPFC (control 83.57 ± 10.8 cells mm^{-2}, stress 83.41 ± 12.8 cells mm^{-2}), HIP (control 63.32 ± 3.82 cells mm^{-2}, stress 66.40 ± 3.46 cells mm^{-2}), M1 (control 207.3 ± 25.0 cells mm^{-2}, stress 211.0 ± 17.2 cells mm^{-2}) or S1 (control 217.9 ± 12.8 cells mm^{-2}, stress 184.4 ± 26.9 cells mm^{-2}) (Supplementary Figure 4c; $P > 0.05$, *t*-test in each area). These data suggest that MS alone is not sufficient to induce the loss of PV cells. There were also no significant differences in the densities of PV-negative and GAD67-positive cells in these areas
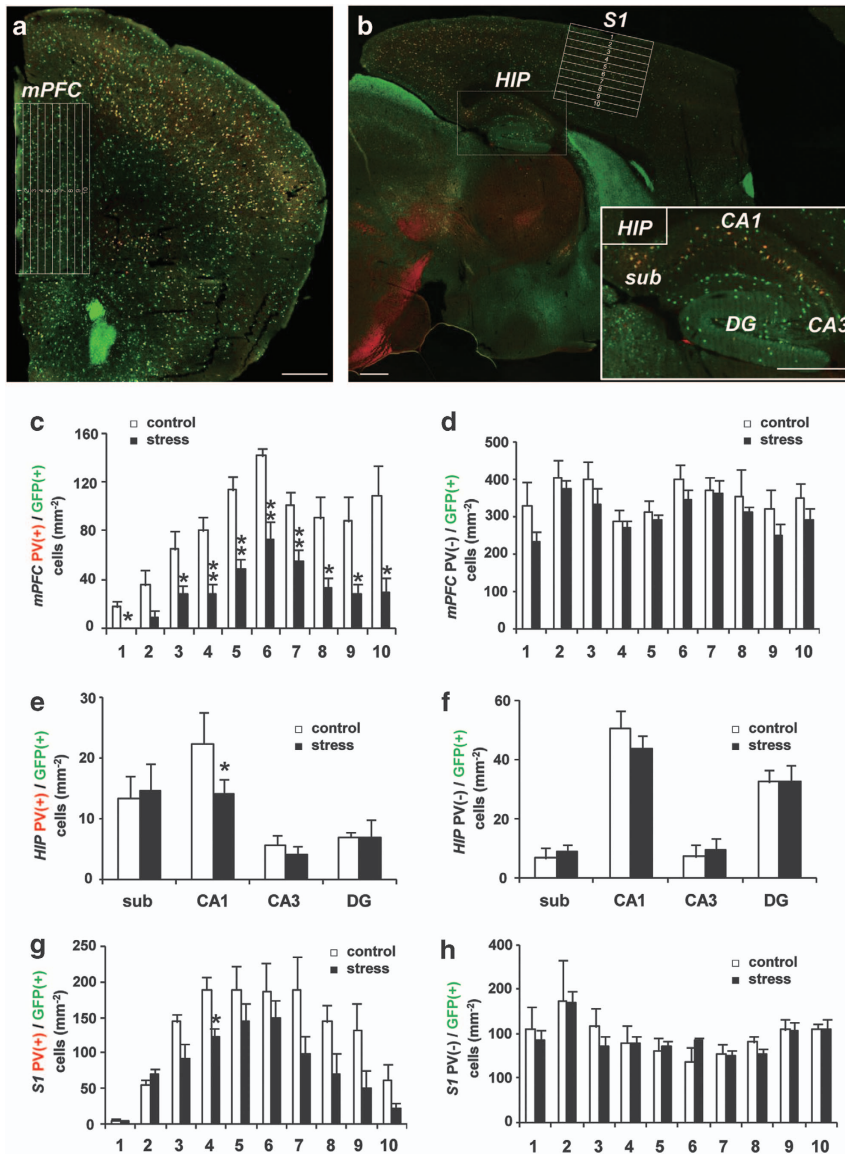
Figure 5. Topography of the reduction of parvalbumin (PV)-positive γ-aminobutyric acidergic (GABAergic) interneurons following maternal stress (MS) in P21 GAD67[+/GFP] mice. (**a**) Coronal sections of the medial prefrontal cortex (mPFC) were divided into 10 zones. (**b**) Sagittal sections of the hippocampus (HIP) were further divided into subregions, and the somatosensory cortex (S1) was divided into 10 zones. Bars in **a** and **b** = 500 μm. (**c–h**) Quantitative analysis of densities of PV (+)/GFP (+) cells (**c, e, g**) and PV (−)/GFP (+) cells (**d, f, h**) (*$P < 0.05$, **$P < 0.01$, t-test). (**c, d**) mPFC: control, $n = 5$ sections from three brains; stress, $n = 5$ sections from four brains. (**e, f**) HIP: control, $n = 5$ sections from three brains; stress, $n = 4$ sections from four brains. (**g, h**) S1: control, $n = 4$ sections from three brains; stress, $n = 5$ sections from four brains.

(Supplementary Figure 4d; mPFC, control 194.5 ± 14.3 cells mm^{-2}, stress 204.2 ± 20.5 cells mm^{-2}; HIP, control 127.2 ± 27.9 cells mm^{-2}, stress 120.1 ± 13.2 cells mm^{-2}; M1, control 134.4 ± 16.5 cells mm^{-2}, stress 115.8 ± 13.2 cells mm^{-2}; S1, control 116.4 ± 15.3 cells mm^{-2}, stress 127.4 ± 13.4 cells mm^{-2}; $P > 0.05$, t-test in each area).

DISCUSSION

MS had a specific impact on the proliferation of PV-positive GABAergic neurons in the GAD67[+/GFP] fetal brain. The major findings presented here are the following: (1) neurogenesis of GABAergic neurons in the fetal MGE was suppressed during MS, (2) most proliferating cells in the MGE expressed GRs whose activation appeared to affect cell proliferation and (3) the number of PV-positive GABAergic cells in P21 mice stressed during the fetal period was significantly decreased in brain regions considered to be involved in psychiatric illness.[41] In addition, (4) in

contrast to GAD67[+/GFP] mice, GAD67^{+/+} pups did not show a loss of PV-positive GABAergic cells following MS. Stress during the gestational period[2,6] and GAD67 abnormalities[42,43] are both considered to be risk factors for psychiatric disorders that may involve perturbation of neurogenesis.[44] Thus, our model reproduced both the risks and anatomical outcomes observed in schizophrenia patients, such as a decrease in the number of PV-positive GABAergic interneurons.[45,46]

Under stressful conditions during pregnancy, maternal glucocorticoid concentrations can reach high levels. Although most glucocorticoid is transformed into an inactive form by the placental enzyme 11β-hydroxysteroid dehydrogenase,[47] if placental glucocorticoid levels become extremely high, fetal glucocorticoid levels can increase and may affect development.[48] In our study, maternal serum CORT levels increased twofold following MS, as did fetal CORT levels[29] (Supplementary Figures 1c and d). Thus, the CORT discharged from the maternal adrenal cortex

during stress may have been transferred to the fetus through the placenta, thereby perturbing brain development through GRs.

CORT influences the proliferation of embryonic neuronal stem cells by activating GRs.[49] Our results show that proliferating cells in the MGE have GRs, but few such cells were observed in the VZ (Figure 3b). Thus, CORT increases by MS could exclusively inhibit the proliferation of GABAergic progenitors expressing GRs. Furthermore, in our previous study, fetal CORT levels in GAD67[+/GFP] mice were significantly higher than those in GAD67^{+/+} mice with or without MS.[29] Therefore, heterozygosity of the *Gad1* gene encoding GAD67 may influence CORT levels during MS. In addition, GAD67 and ambient GABA may provide an essential environment for neurogenesis of GABAergic neurons,[50–53] because exogenous DEX administration had a rather subtle impact on GAD67^{+/+} fetuses (Figure 3b). Decreased GAD67 expression is increasingly recognized in the postmortem brains of schizophrenia patients and other major psychiatric diseases[22,24,42,54–56] including autism.[57] Thus, *GAD1* abnormalities may be a genetic risk factor that could interact with environmental risk factors such as MS to generate psychiatric disorders.[58,59]

In contrast to our results, administration of an exogenous stress hormone retarded the radial migration of CP cells.[60] As demonstrated in Figures 1 and 2, neither radial migration nor tangential migration was altered by exposure to MS. Intrinsic stress hormones may have less of an effect on GRs compared with exogenous synthetic compounds. In addition, in our results, lower expression of GRs was observed in proliferating cells from the VZ and in other neocortical regions when compared with the MGE (Figure 3). Thus, retardation of radial migration may require higher concentrations of glucocorticoids.[60] In addition, a recent study reported impaired tangential migration following MS at the earliest time point (E12).[61] Thus, the influence of MS may depend on the period of exposure, for example, MS at later gestational stages, such as in our model, does not affect tangential migration but affects the generation of GABAergic neurons.

Decreased PV mRNA levels appear to be common in schizophrenia patients.[62,63] The present study demonstrates for the first time a decrease in GABAergic neurogenesis in fetuses and loss of PV-positive interneurons in P21 mice exposed to MS during the fetal period. In schizophrenia patients, decreased neuronal PV mRNA expression is highly associated with a decrease in the density of neurons expressing GAD67 mRNA, and only half of PV mRNA-positive neurons were found to have detectable levels of GAD67 mRNA.[64] In the present study, the population of GABAergic interneurons generated during MS decreased. Of these cells, the number of PV-positive GABAergic interneurons was significantly decreased in the mPFC, HIP and S1 of GAD67[+/GFP] mice but not GAD67^{+/+} mice. Furthermore, PV-negative GABAergic interneurons were not affected in both genotypes. These results indicate that exposure to MS, in addition to heterozygous deletion of *Gad1*, exclusively has an impact on the proliferation of GABAergic neuronal precursors fated to be PV-positive.

Some genetic model animals of schizophrenia show decreases in PV-positive neurons in the mPFC.[65] Interneuron subgroups have distinct spatial and temporal origins, with cortical PV-expressing interneurons originating primarily within the MGE[66] and notably at approximately E15.[67] In our model, proliferation during E15.0 to E17.5 in the MGE was significantly decreased (Figure 2) by MS applied during that period, suggesting that the generation of PV-expressing neurons destined to migrate into the mPFC, the CA1 of the HIP and layer III of the S1 was disturbed. Cortical and hippocampal PV-positive interneurons have a crucial role in cognitive function and working memory.[68–70] Therefore, impairment of these cells could underlie working memory impairment in schizophrenia patients.[71,72]

MS applied to the mothers of GAD67-heterozygous fetuses caused damage to the embryonic brains, especially to precursor

cells destined to be PV-positive GABAergic interneurons in particular cortical regions, which recapitulates the morphological abnormality observed in psychiatric disorders. Considering that GAD67 homozygous offspring did not show such outcomes following MS, this finding could explain the selective loss of PV-positive GABAergic interneurons observed in specific brain regions in patients with GAD67 abnormalities exposed to MS. Thus, by providing evidence for an interactive effect of *GAD1* and MS on the abnormal phenotype of PV neurons, the present results provide mechanistic insight into the interactions between genetic and environmental risk factors in the etiology of schizophrenia and other psychiatric disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)